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IMPORTANCE OF CALCIUM FOR THE POTASSIUM EXCHANGE OF THE TUMOUR CELL.

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THE energy-metabolism of the tumour-cell, both fermentation and respiration, depends to some extent upon the presence of potassium and calcium in the medium. This was shown in a series of investigations carried out with the Jensen rat sarcoma and the Flexner-Jobling rat carcinoma (Lasnitzki, 1928, 1933, 1934, 1936 ; Lasnitzki and Rosenthal, 1929, 1933, 1935). In a medium which contained a physiological amount of potassium or calcium the rate of metabolism was found to be greater than in the control medium containing sodium as a single cation, and this increase was more pronounced if potassium and calcium were present together.

It may be suggested as an explanation for this synergistic effect that the true activator of the energy-metabolism is potassium, while the action of calcium is due to an inhibition of the diffusion of potassium from the tumour-cell into the medium. This explanation would appear to be supported by the fact that calcium, in a potassium-free medium, was unable to act after the tissue had been kept for a while in contact with the control medium. On the other hand, the substitution of potassium for calcium resulted in definite action (on fermentation). But the assumption should also prove correct if potassium is present, in view of the fact that the rate of metabolism in the medium with both cations was greater than in the medium with potassium alone. The latter case, however, presupposes that the intra-cellular potassium of the tumour tissue as taken from the body is not in equilibrium with the potassium of the medium.

This conception of the action of calcium appears to agree with the results of investigations in which the potassium content of tumour-tissue after suspension in media with and without calcium was estimated.

Methods.

The media were similar to those utilized in fermentation experiments (Ringer's solutions containing NaCl, $NaHCO_3$ and glucose, apart from KCl and $CaCl_2$). In each experiment the fresh tissue of the Jensen rat sarcoma

(marginal part) was cut into a large number of thin slices, which were suspended, for several hours at room temperature, in the media concerned. The slices were then rinsed rapidly in distilled water, dried and finally ashed after estimation of the dry weight. A solution of the ash served for the estimation of potassium.

Results.

First, it was found that most of the potassium present in the tumour tissue was freely diffusible, the potassium content of tissue after suspension in a medium without potassium and calcium being, on an average, about 85 p.c. lower than that of non-suspended tissue. The reduction of the potassium content was still considerable, although less, if the medium contained a physiological amount of potassium (0·0025 *M* KCl). This shows that the supposition considered above is correct.

Potassium Content of Tumour Tissue (Jensen Rat Sarcoma) after Suspension:*

a: *In Media Without Potassium.*

Experiment number.	Mg. of potassium per 100 mg. dry weight of tissue.	
	Medium without calcium.	Medium with calcium.
1	0·35	0·65
2	0·25	0·90
3	0·55	0·70
4	0·40	0·45
5	0·25	0·55
6	0·30	0·50
7	0·25	0·40
8	0·15	0·35
9	0·35	0·55
10	0·20	0·40
Average	0·31	0·55

b: *In Media With Potassium.*

Experiment number.	Mg. of potassium per 100 mg. dry weight of tissue.	
	Medium without calcium.	Medium with calcium.
1	0·40	1·50
2	0·30	0·65
3	0·20	1·15
4	0·75	0·90
5	0·60	1·15
6	0·40	1·30
7	0·40	1·35
8	0·40	1·45
Average	0·43	1·18

* Average time after inoculation: Group "a" about 22, Group "b" about 20 days.

The effect of the addition of calcium to the medium was studied, the results obtained in two groups of experiments being shown in the table. In group "a" the medium contained no potassium, and we see that the potassium content of the tissue was, on an average, increased by about 80 p.c. if calcium (0·0018 *M* $CaCl_2$) was present in the medium. In group "b" the medium contained potassium (0·0025 *M* KCl), and here the effect caused by the addition of calcium appears to be, generally, more pronounced, both absolutely and relatively. It will be seen that the average potassium content of the tissue after suspension in the control medium was somewhat greater than in the former group, but nevertheless the average potassium content increased by about 175 p.c. if the medium contained calcium in the concentration used as

above. The average time during which the tissue had been kept in contact with the media was, approximately, 3½ hours in both groups. It is to be noted that the actual potassium content of the tissue after suspension in the medium with both potassium and calcium was, on an average, still about one-third lower than that of the non-suspended tissue.

These results certainly indicate that calcium is capable of inhibiting the diffusion of potassium from the tumour-cell into the medium, and it is to be presumed that it would act in the same manner if diffusion was taking place in an opposite direction. Further, it may be concluded that similar happenings occur *in vivo*. Thus calcium may play an important part in the exchange of potassium between the tumour-cell and its naturally surrounding medium, and therefore in the regulation of the former's energy-metabolism and growth.

SUMMARY.

Estimations of potassium in tumour tissue (Jensen rat sarcoma) which had been suspended in Ringer's solutions, with and without calcium, indicate that calcium is capable of inhibiting the diffusion of potassium from the tumour-cell into the medium, and, possibly, also the same diffusion in an opposite direction.

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FURTHER STUDIES OF COMPLEMENT-FIXATION IN INFLUENZA: ANTIGEN PRODUCTION IN EGG-MEMBRANE CULTURE AND THE OCCURRENCE OF A ZONE PHENOMENON.

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THE authors (Fairbrother and Hoyle, 1937; Hoyle and Fairbrother, 1937*a*) demonstrated that the sera of influenza convalescents and of many normal persons are able to fix complement specifically in the presence of an antigen prepared from desiccated mouse lungs infected with virus.

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In subsequent studies (Hoyle and Fairbrother, 1937*b*) evidence was brought forward to indicate that the reacting antigen in virus suspensions was not the influenza virus itself, but a soluble substance probably produced during multiplication of the virus in the tissues. It was therefore expected that this substance would be produced during growth of the virus in tissue-culture. Smith (1936) has described complement-fixation with culture virus as antigen, but the amount of antigen present in his cultures was apparently very small, as fixation was only demonstrated by prolonged contact of the reagents ; the virus was cultivated on a medium composed of minced chick embryo and Tyrode's solution. We have been able to obtain more satisfactory results by culture on the chorio-allantoic membrane of the hen's egg ; the findings are recorded in this paper.

PRODUCTION OF ANTIGEN IN CULTURE.

Ten- to twelve-day old fertile eggs were inoculated with 0·1 c.c. of a washed elementary body suspension of the W.S. strain of influenza virus. The suspension was prepared as described previously (Hoyle and Fairbrother, 1937*b*) and did not contain any demonstrable complement-fixing antigen. The technique of culture was that described by Burnet (1936). After 48 hours the eggs were opened, and the chorio-allantoic membranes removed and desiccated *in vacuo* over calcium chloride.

The dried membranes were weighed and 2 p.c. extracts prepared by grinding with sand and saline and centrifuging at 4000 r.p.m. for 30 minutes. The extracts were then tested for antigen content. Falling dilutions of antigen were used with a constant amount of complement (2½ M.H.D.) and a constant amount of serum. The positive serum was from a human convalescent ; its titre was 1 : 32 and it was used diluted 1 : 8. A known negative human serum was used as control. Fixation was for 1 hour at 37° C. Normal uninoculated membranes were treated similarly and used as controls. The results are shown in Table I. The antigen content of the infected membranes compared favourably with that of infected mouse lungs, while the normal membranes gave no fixation. Equally satisfactory results were obtained when fresh membranes were extracted with saline, desiccation being omitted ; such saline extracts could be stored for some weeks at 0° C. if a few drops of chloroform were added as preservative. These culture-virus antigens were found to be almost devoid of anti-complementary properties.

Smith (1935) considered that the virus could not be cultivated with certainty on the chorio-allantoic membrane, but in our work no difficulty has been experienced, the virus multiplying regularly both on first inoculation and on serial passage. The membranes usually showed no changes as a result of growth of the virus, occasionally small opaque spots were seen, but these were not regularly present and were possibly caused by mechanical injury to the membrane during inoculation.

It was thought possible that antigen production might be increased if virus were passed serially in culture, and experiments were done to determine this. Virus was passed in series from egg to egg, and at each passage six

TABLE I.—*Production of Complement-Fixing Antigen in Egg-Membrane Culture.*

Antigen.	Serum.	Antigen dilutions: 1 : 1.	1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 64.
2 p.c. mouse lung antigen .	Positive .	0	0	0	0	+	+++	++++
	Negative .	+++	++++	++++	++++	++++	++++	++++
Infected membrane (1) .	Positive .	0	0	0	0	0	+++	++++
	Negative .	++++	++++	++++	++++	++++	++++	++++
„ „ (2) .	Positive .	0	0	0	0	0	+	++++
	Negative .	++++	++++	++++	++++	++++	++++	++++
„ „ (3) .	Positive .	0	0	0	0	0	0	+++
	Negative .	++++	++++	++++	++++	++++	++++	++++
„ „ (4) .	Positive .	0	0	0	0	+	++++	++++
	Negative .	++++	++++	++++	++++	++++	++++	++++
Normal membrane (1) .	Positive .	++++	++++	++++	++++	++++	++++	++++
	Negative .	++++	++++	++++	++++	++++	++++	++++
„ „ (2) .	Positive .	++++	++++	++++	++++	++++	++++	++++
	Negative .	++++	++++	++++	++++	++++	++++	++++

++++ = Complete hæmolysis. +, ++, +++ = Intermediate degrees of hæmolysis. 0 = No hæmolysis.

membranes were taken, extracted with 6 c.c. of saline, centrifuged, and the supernatant fluid preserved at 0° C. with addition of chloroform. The preparations were then tested for antigen content as in the previous experiment. The results are shown in Table II. Antigen production was not noticably increased by serial passage.

TABLE II.—*Effect of Serial Passage on Production of Complement-fixing Antigen in Egg-Membrane Culture.*

Antigen.	Antigen dilutions.							
	1 : 1.	1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 64.	1 : 128.
1st passage	0	0	0	0	+	+++	++++	++++
2nd ,,	+	0	0	0	0	+	++	++++
3rd ,,	+	0	0	0	0	+	+++	++++
4th ,,	0	0	0	0	+	+++	++++	++++
5th ,,	+	0	0	0	0	+	+++	++++

Antigen controls all ++++. Serum controls ++++. ++++ = Complete hæmolysis. +, ++, +++ = Intermediate degrees of hæmolysis. 0 = No hæmolysis.

ZONE PHENOMENON IN THE FIXATION TEST.

With the potent antigens obtained from infected egg membranes a phenomenon has been observed which was not seen with the less concentrated and more anti-complementary antigens derived from mouse lung. This is a tendency to zoning in antigen titrations. The phenomenon is partially seen with the more active antigens in Table II, where fixation was incomplete in the first tubes, in which the antigen was in greatest concentration. A more definite picture is shown in Table III. In this experiment, falling dilutions of the antigen were tested against falling dilutions of the serum. Definite zoning is seen in the 4th, 5th and 6th horizontal rows. With minimal amounts of antibody fixation does not occur, or is incomplete in the region of antigen excess. The converse phenomenon, namely failure of fixation with antibody excess, has not been encountered, possibly because sera with a sufficiently high antibody content have not been available.

TABLE III.—*Zone Phenomenon in Complement-fixation Test.*

Serum dilution.	Antigen dilution.						
	1 : 1.	1 : 2.	1 : 4.	1 : 8.	1 : 16	1 : 32.	1 : 64.
1 : 2	0	0	0	0	0	0	+++
1 : 4	0	0	0	0	0	+	++++
1 : 8	0	0	0	0	0	+	++++
1 : 16	+	0	0	0	0	++	++++
1 : 32	++++	+	0	0	0	++++	++++
1 : 64	++++	++++	++++	+	+	++++	++++
1 : 128	++++	++++	++++	++++	++++	++++	++++

++++ = Complete hæmolysis. +, ++, +++ = Intermediate degrees of hæmolysis. 0 = No hæmolysis.

DISCUSSION.

The results recorded above demonstrate clearly that complement-fixing antigen is produced when the influenza virus is cultivated on the chorio-allantoic membrane of the developing hen's egg. A saline extract of either the fresh or dried infected membrane forms a very satisfactory antigen for use in the fixation test, as it has a high antigen content and little anti-complementary properties.

Francis and Magill (1937) have demonstrated that the influenza virus can be directly isolated from man by culture on egg membranes, but the practical use of this method of isolating virus was limited owing to the fact that it was impossible to demonstrate the presence of virus in the inoculated membranes except by the inoculation of animals. Our results indicate that the presence of virus in the membranes can be shown simply and with certainty by demonstrating the presence of complement-fixing antigen. Hyde and Chapman (1937) have also been able to isolate the virus directly from man by culture on egg membranes, and they consider that the presence of virus in the membranes can be recognized by the occurrence of definite pathological changes. In this respect their experience differs from that of other workers, who, like ourselves, have not found any constant pathological appearances in the infected membranes.

The occurrence of the zone phenomenon in complement-fixation reactions is well known, the phenomenon is important and indicates the need for accurate antigen titration, since the use of excess antigen may greatly reduce the sensitivity of the test. The antigen should be titrated against small doses of antibody and the central tube of the zone of fixation taken as the optimal antigen concentration.

SUMMARY.

1. Complement-fixing antigen is produced when the influenza virus is cultivated on the chorio-allantoic membrane of the developing hen's egg.

2. A saline extract of either fresh or dried infected egg membrane forms an excellent antigen for use in the fixation reaction.

3. The occurrence of zone phenomena in the influenza complement-fixation reaction is described.

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ACTIVE IMMUNIZATION AGAINST EXPERIMENTAL INFLUENZA: THE USE OF HEAT-KILLED ELEMENTARY BODY SUSPENSIONS.

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IT has been repeatedly shown that mice can be completely, and ferrets partially, immunized against influenzal infection by the injection of living or formolized virus (Smith, Andrewes and Laidlaw, 1935; Shope, 1936; Andrewes and Smith, 1937). Attempts have also been made to immunize man, but the results obtained up to the present have been inconclusive. An increase in antibody titre has resulted after vaccination, but protection against infection has not been definitely demonstrated. It has recently been shown that the influenza virus has a complex antigenic structure (Fairbrother and Hoyle, 1937), and it seemed an important point to determine the most suitable type of antigen for use in vaccination. Studies have therefore been made of the immunogenic properties of various preparations; the results are recorded in this paper.

THE IMMUNOGENIC ANTIGEN.

It has been previously demonstrated (Hoyle and Fairbrother, 1937) that crude suspensions of the influenza virus can be separated into two major fractions:

(1) The elementary bodies.
(2) A soluble substance apparently derived from the bodies, which reacts in the complement-fixation test.

Preparations of these two fractions were made in the following manner, and their infectivity and immunizing activity determined.

Elementary body suspensions were prepared from infected mouse-lungs by the technique of differential centrifugation previously described (Hoyle and Fairbrother, 1937).

The "*soluble substance*" was prepared by extracting desiccated infected mouse-lungs with saline and centrifuging at 13,000 r.p.m. for 1 hour. The supernatant fluid was treated with 0·025 p.c. acetic acid, and the resulting precipitate removed by low-speed centrifugation and redissolved in phosphate-buffered saline (pH 7·4). The solution was finally cleared of residual elementary bodies by centrifuging at 13,000 r.p.m. for one hour.

The infectivity of the fractions was tested by instilling 4–6 drops intranasally into groups of 10 mice under ether anæsthesia; 4 days later the mice were killed and examined for pulmonary lesions (Table I).

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TABLE I.—*Properties of Fractions of the Influenza Virus.*

	Elementary bodies.	Soluble substance.	Original suspension.
Infectivity	+++	0	+++
Presence of complement-fixing antigen	0	+++	+++
Protecting power in mice	+++	0	+++
Stimulation of antibodies in mice—			
(*a*) Neutralizing	+++	0	+++
(*b*) Complement-fixing	+	+−	++

Key.—0 = no action. +++ = marked action. +−, +, ++ = intermediate grades.

In examination of the immunizing activity, groups of 30 mice each were vaccinated with the two preparations, two doses of 0·2 c.c. being given at intervals of 7 days, the first dose subcutaneously, and the second intraperitoneally. One week later 15 mice from each group were tested for immunity by the intranasal inoculation of approximately 10,000 minimal infecting doses of active virus. The remaining mice were bled and the pooled sera of each group tested for the presence of neutralizing and complement-fixing antibodies (Table I).

The soluble substance did not induce any appreciable antibody formation although it proved an admirable reaction agent in the complement-fixation test ; it was therefore not surprising to find that it did not immunize the mice. In previous work (Hoyle and Fairbrother, 1937) we had obtained complement-fixing antibody by injecting a crude preparation of complement-fixing antigen which was not completely free from elementary bodies ; while the injection of washed elementary bodies failed to produce this antibody. This would suggest that the soluble substance is antigenic when associated with elementary bodies, but when prepared in a relatively pure form it is nor antigenic ; *i.e.* it behaves like a haptene.

The elementary bodies, which undoubtedly represent the actual virus, immunized the mice and also induced antibody production. In previous work with rabbits the inoculation of washed elementary bodies had induced the formation of neutralizing, but not complement-fixing, antibodies. In the case of mice, however, the inoculation of washed elementary bodies resulted in the production of neutralizing and, to a less degree, complement-fixing antibodies, although the elementary body suspension did not fix complement *in vitro*. A possible explanation of this result is that in the susceptible animal some multiplication of virus occurred in the tissues with production of complement-fixing antigen, while in the non-susceptible rabbit multiplication did not take place.

The results indicated that the most suitable preparation of the virus for prophylaxis was the elementary body suspension. Such suspensions have definite advantages over more crude preparations as they are to a large extent free from extraneous protein material.

ELEMENTARY BODY VACCINES.

It was decided to examine the immunizing power of elementary body suspensions after various forms of treatment. The following methods were used :

(1) Addition of formaldehyde (1 : 1000).
(2) ,, ,, (1 : 5000).
(3) ,, phenol (0·5 p.c.).
(4) Heating for 30 minutes at 45° C.
(5) ,, ,, 50° C.

The suspensions were kept overnight in the ice-box before use.

The infectivity of these preparations was first tested ; groups of 4 mice being inoculated intranasally with the different vaccines (Table II). The usual dose of the original suspension contained approximately 10,000 minimal infecting doses. Treatment with 0·5 p.c. phenol had no apparent effect on the virus, heating at 45° C. caused some inactivation, while at 50° C. the virus was almost completely inactivated. The addition of 1 : 1000 formaldehyde inactivated the virus completely, while 1 : 5000 formaldehyde did not entirely destroy the virus, though it was found that on further standing at 0° C. the virus was slowly killed.

TABLE II.—*Infectivity Tests of the Vaccines.*

Type.	Result.			
Living (untreated) .	D_4 .	D_4 .	++ .	++
Phenol 0·5 p.c. .	D_4 .	D_4 .	++ .	++
Formaldehyde 1 : 1000	0 .	0 .	0 .	0
,, 1 : 5000	+− .	+− .	0 .	0
Heated at 45° C. .	++ .	+ .	+ .	+−
Heated at 50° C. .	+− .	0 .	0 .	0

Key.—Each symbol represents a mouse. D_4 = death on the 4th day with extensive pulmonary lesions. ++ = killed on the 4th day with extensive pulmonary lesions. + = killed on the 4th day with marked unilateral pulmonary lesions. +− = killed on the 4th day with slight unilateral pulmonary lesions. 0 = killed on the 4th day but pulmonary lesions not found.

The immunizing power of these various vaccines was then determined by inoculating mice with two doses of 0·2 c.c. at 7-day intervals, the first dose being given subcutaneously and the second intraperitoneally. The mice were tested 7 days later by intranasal inoculation of virus, one half receiving 2000 minimal infecting doses, and the remainder 100 minimal infecting doses. The result is shown in Table III.

TABLE III.—*Elementary Body Preparations : Immunogenic Action in Mice.*

	Virus (2000 m.i.d.).						Virus (100 m.i.d.).				
	D.	++.	+.	+−.	0.		D.	++.	+.	+−.	0.
Living . . .	0	0	1	2	9	.	0	0	0	1	10
Formolized (1/1000) .	0	0	0	3	9	.	0	0	0	1	10
,, (1/5000) .	0	0	1	2	9	.	0	0	0	2	8
Phenolized (1/200) .	0	0	0	4	8	.	0	0	0	2	9
Heated (50° C.) . .	0	0	3	2	7	.	0	0	0	1	11
Controls . . .	7	3	1	0	0	.	3	4	4	1	0

D. = Death within 4 days with extensive lesions. ++ = extensive bilateral lung lesion. + = Extensive unilateral lung lesion. +− = Small unilateral lung lesion. 0 = No lesion.

All the preparations conferred considerable protection as the majority of the mice in each group exhibited a high degree of immunity. Phenol (0·5 p.c.) had apparently no appreciable effect on the infectivity of the virus, and it was consequently considered that it would serve as an excellent preservative for the vaccines. The results obtained with the heated vaccine were particularly interesting, as it is generally considered that heat-killed viruses possess little or no immunogenic power. In the above experiment the virus was not completely destroyed at 50° C., the activity of the heated suspensions was therefore examined in more detail.

Heated elementary body suspensions.—Further tests were carried out to determine whether protection could be obtained in mice by means of heat-killed elementary body suspensions. Suspensions, heated at 49½–50° C. for 30 minutes and with 0·5 p.c. phenol added, were examined for infectivity and immunogenic activity on several occasions. The results are arranged in Table IV.

TABLE IV.—*The Activity of Elementary Body Suspensions heated at 50° C. for ½ Hour.*

Experiment.	Infectivity.	Induction of protection in mice.
A	+−, 0, 0, 0,	Yes.
B	+−, 0, 0, 0	,,
C	0, 0, 0, 0, 0, 0	,,
D	+−, 0, 0, 0, 0, 0	,,
E	+−, +−, +−, +−, 0, 0, 0, 0	,,

In all cases the unheated suspension contained approximately 10,000 m.i.d. in the standard dose, and produced marked lesions in all mice with a mortality-rate of about 40 p.c. + = Mouse with small unilateral lesions. 0 = Mouse with no lesion. Each figure represents one mouse.

The infectivity tests indicate that by heating at about 50° C. for 30 minutes the virus is not always completely inactivated. But while some infectivity may remain, much of the virus is undoubtedly destroyed. It is, however, important to note that this destruction does not appear to be accompanied by any appreciable loss in immunizing activity of the vaccines.

The relative values of vaccines heated at 50° C. and 57° C. were next compared. An elementary body suspension was divided into two portions, one being heated at 50° C. for 30 minutes, and the other at 57° C. for the same time. Both were phenolized and tests for infectivity and immunizing action were carried out in the usual manner (Table V).

TABLE V.—*Comparison of Elementary Body Suspensions heated at 50° C. and 57° C.*

A. Infectivity:	Result.
57° C.	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0
50° C.	+−, +−, +−, +−, +−, +−, 0, 0, 0, 0, 0, 0
Controls	D_3, D_4, ++, ++

B. Immunizing Activity:

	Result (test virus = 1000 m.i.d.).				
	D.	++.	+.	+−.	0.
57° C.	0	0	0	0	9
50° C.	0	0	0	1	8
Controls	1	3	3	4	0

Significance of symbols as in previous tables.

Although the vaccine heated at 57° C. was apparently completely non-infective, it produced a degree of protection comparable with that given by the incompletely inactivated virus. The protecting power of elementary body vaccines heated at 57° C. has been confirmed in several subsequent experiments. It follows from the results recorded above that heating at 57° C. did not appreciably impair the immunizing capacity of the elementary bodies. Moreover there was no alteration in the physical character of the suspension. This is in marked contrast with the effect of heat at 57° C. on the crude virus suspension, when there is some protein coagulation together with a change from a red opalescent fluid to a brown turbid suspension. Furthermore, a preliminary experiment suggests that the immunizing action of heated crude suspensions is very irregular when given in the usual dosage. It is therefore important to emphasize that the use of heat at 57° C. to inactivate the virus is satisfactory only with elementary body suspensions.

Source of virus for use in vaccines.—As material of animal origin has many disadvantages for human application, it has been suggested that virus for such purposes should be obtained by cultivation in artificial media (Francis and Magill, 1937). We therefore prepared elementary body suspensions from cultures of virus on the chorio-allantoic membrane of 10–12-day chick embryos, but found that the yield of virus was very small compared with that obtainable from infected mouse lungs. The preparations also appeared to be less immunogenic in mice, due probably to their smaller virus content. It was consequently considered that the preparation of elementary body suspensions from culture material was unsatisfactory for use on a large scale as a considerable amount of material and much labour were required to produce a sufficient yield of virus.

IMMUNIZATION OF FERRETS.

The complete protection of ferrets by artificial immunization has proved more difficult than the immunization of mice. Smith, Andrewes, Laidlaw (1935) found that both live and formolized virus given subcutaneously produced only partial immunity, and Shope (1936) found with swine virus that vaccination by the subcutaneous route was only successful when the virus used was of ferret origin, though both mouse and swine virus were effective by the intraperitoneal route.

Our own experiments with ferrets have also been unsatisfactory. Living and heated (50° C.) suspensions were injected into two groups in two doses, the first subcutaneously and the second intraperitoneally (doses of 1·0 c.c.). The ferrets, together with a group of convalescent animals, were then tested for protection by the intranasal inoculation of a strain of virus adapted to the ferret. The results are shown in Table VI.

TABLE VI.—*Immunization of Ferrets.*

Type of vaccine.	Number of animals.	Result: Number developing clinical symptoms.
Heated 50° C. . . .	6	6
Live	3	3
,, (convalescent 3–4 weeks)	3	0
,, (normal) . . .	6	6

It will be seen that neither the live nor the dead virus had any demonstrable protecting effect; there was no significant difference in the clinical symptoms and temperature responses of the vaccinated and control animals. Serological tests were not carried out, but in previous work it had been found that vaccination with living suspensions produced a partial immunity as indicated by a definite antibody response. In contrast to this, the ferrets recently recovered from the typical infection were apparently unaffected by the same dose of virus; vaccination thus appeared to confer a much lower degree of immunity in ferrets than did infection.

DISCUSSION.

The results recorded above indicate that elementary body suspensions prepared by differential centrifugalization contain the effective immunogenic fraction of the influenza virus, and are capable of inducing complete immunity in mice. These preparations are largely free from extraneous protein and would therefore be more suitable for use in human prophylaxis than the cruder preparations which have been previously employed.

Although live virus has been employed in human prophylaxis without ill effect (Francis and Magill, 1937), the experience of Shope (1936) with the swine virus suggests that the use of live virus may not be without danger. Shope considered that the appearance of swine influenza in two herds of swine shortly after vaccination with live virus was attributable to the vaccine. Our results show that in the experimental animal elementary body suspensions killed by heating at 57° C. for 30 minutes, and preserved by addition of 0·5 p.c. phenol, have an immunizing power equal to that of live virus. Such suspensions would therefore seem most suitable for human use.

It is to be noted that while these suspensions are satisfactory prophylactics in mice, the immunization of ferrets appears to be much more difficult. As the disease in ferrets resembles the human disease much more closely than does the disease in mice, it might be thought that the outlook for human preventive vaccination was poor. It must, however, be remembered that in testing for immunity in ferrets the animal is subjected to the intranasal inoculation under ether anæsthesia of an enormous dose of virus, a test of a severity which would never be encountered under natural conditions by man.

SUMMARY.

1. The immunogenic factor in virus suspensions is the elementary body.

2. Elementary body suspensions are more easily and satisfactorily prepared from infected mouse-lungs than from cultures of the virus on the chorio-allantoic membrane of the developing chick embryo.

3. Elementary body suspensions, inactivated by heat at 57° C. for ½ hour, injected subcutaneously and intraperitoneally into mice induce a marked degree of immunity.

4. Such suspensions appear to be as effective as living or formolized virus.

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STUDIES ON THE SEROLOGICAL INTER-RELATIONSHIPS OF THE RABBIT VIRUSES, MYXOMATOSIS (SANARELLI, 1898), AND FIBROMA (SHOPE, 1932).

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A brief interim report of the main results obtained in the course of a comparative serological study of the elementary bodies of myxoma and fibroma, based on agglutination tests, was submitted by the writer to the Virus Section of the Second International Congress for Microbiology in July, 1936 (Ledingham, 1937). This study was undertaken with the object of throwing some light on the mechanism of the protection which the fibroma-recovered animal enjoys from the otherwise almost invariably fatal issue resulting from infection with the virus of myxoma. The demonstration of this fact we owe to Shope (1932), and in October, 1935, when the writer's experiments were commenced, the problem seemed a more straightforward one than it has since become by reason of the remarkable experimental findings of Berry and Dedrick (1936) which postulate a real conversion of fibroma to myxoma *in vivo* when the former virus is administered in the "living" state in conjunction with the latter rendered inactive by heat treatment. Any inquiry into the serological relationships of myxoma and fibroma on lines previously exploited by the writer and his colleagues in the study of other virus infections, had, at any rate in October, 1935, to contend with the fact that recovery from experimental infection with myxoma was of the rarest occurrence and, consequently, that in order to secure sera from animals with a history of recovery from myxoma infection, it would be necessary to infect them in the first instance with the virus of fibroma. This procedure was, on *a priori* grounds at least, liable to introduce complications from the strict serological standpoint, but fortunately, through the

kindness of Sir Charles Martin, who, in the course of his very successful efforts to spread myxoma infection among rabbits in open enclosures by introducing among them experimentally infected animals (Martin, 1936), observed recovery to take place in a very few cases, I was able at the outset of this work to secure blood from five such recovered animals and at a later period to have three such transferred to the Institute's myxoma room.

In the course of my own work with myxoma I have observed only one recovery from this disease and, strangely enough, this particular animal was one which, about six weeks before it exhibited the typical myxoma syndrome, had been inoculated with myxoma virus heated at 60° C. as a control to a Berry-Dedrick experiment. In all probability it had contracted a natural infection in the myxoma room. Whether the original inoculation of the heated myxoma virus, which apparently was itself impotent, protected the animal in this particular instance from the usual fatal issue, must remain at least very doubtful. Now, since Hurst (1937*b*) has shown that by intracerebral passage of myxoma in rabbits a neuromyxoma variant can be established which, when introduced into skin areas, fails to kill, it is possible to secure at will recovered neuromyxoma animals which are protected from fatal issue when the ordinary virulent form of the virus is introduced. Reference will be made later in the course of this paper to some experiments with this neuromyxoma variant.

MATERIAL AND METHODS.

Source of viruses employed.—The myxoma strains used were the two which had served for Sir Charles Martin's experiments, viz. *A*, received directly from Dr. Aragão, of the Oswaldo Cruz Institute in 1934, and strain *B*, received from Dr. Shope as the one maintained for many years at the Rockefeller Institute, though also hailing originally from the Brazilian Institute. The fibroma virus was received by the writer directly from Dr. Shope as a glycerine-saline testicular sample. At a later stage in the work the inflammatory strain of this virus, as used in experiments by my former colleague, Dr. E. W. Hurst, was also brought into requisition.

Preparation of elementary body suspensions from myxoma and fibroma for micro-agglutination tests.—The methods employed for securing E.B. suspensions of myxoma and fibroma in the purest possible form have followed, in their essentials, lines now generally adopted by virus workers and do not require detailed description. Whatever the initial source of infective material, these lines involve (1) fractional centrifugations, oft repeated, of the crude material from the lesions, aided perhaps by preliminary freezing and thawing if such material is highly cellular ; (2) the deposition of elementary bodies from resulting clarified supernatants by aid of a high-speed centrifuge, sometimes after a preliminary filtration of these supernatants through Berkefeld V filters, though this may involve considerable loss of elementary bodies ; and (3) a final fractional centrifugation of deposited material resuspended in formalin-saline (1 in 400). These final deposits thrown down by the high-speed centrifuge, often in cakes and flakes, demanded, not infrequently, considerable negotiation by trituration before a completely homogeneous and stable suspension of elementary bodies could be secured. Only small quantities, however, of these homogeneous suspensions were necessary for micro-agglutination tests, and, as the work progressed, it was found that such suspensions, frequently tested with known positive sera, retained their agglutinability for several months. This was a great advantage when tests of sera from different animals at different periods after infection had to be made. The most generally used initial myxoma material was the skin-lesions and regional lymph-glands following intradermal inoculation and secured generally from rabbits killed in the moribund state. Use was also made of the exudate from highly œdematous skin-lesions, which were simply cut up into small dice and allowed to drip in the cold. Such fluids were highly potent. A comparative titration of one such exudate fluid and of the serum from the blood of the animal removed at the same time and allowed to clot, showed an I.D. take of 10^{-5} in the former and in the latter of 10^{-1} only.

These exudates naturally required little manipulation before the final high-speed centrifugation.

Another source of material for manipulation which Rivers and Ward (1937) in a recent paper has also used, was the scraped Malpighian skin-layers from animals infected by inunction of virus on the shaved back. Histological study of skin sections from such animals had yielded convincing pictures of the acidophil granules, discrete or in clusters, lying in the cytoplasm of epithelial cells and corresponding with those described by Rivers (1926–7). There can hardly be any doubt that the elementary bodies recovered from the epithelial mass by the manipulative process described and subsequently proved to be the sole infective agents, corresponded with these cytoplasmic granules. No actual infection experiments were performed with deposits thrown down by Baskerville or Ecco types of high-speed centrifuge from Berkefeld V filtrates of scraped epithelial tissue. E.B. suspensions, however, prepared from such deposits behaved in agglutination test like others secured by filtration of material from skin, glands and spleen, and with which infection experiments were carried out. Details of protocols would seem unnecessary. It was clearly shown that such deposits of elementary bodies were infective, while supernatants removed from them were completely depleted of potency. One, therefore, had the assurance that the E.B. material employed for the serological tests really represented the actual virus agents.

For the preparation of E.B. suspensions of the fibroma virus, skin tumour tissue was used from animals sacrificed about the 10th day before superficial necroses manifested themselves.

Preparation of sera.—All sera from bleedings were usually tested on the day of bleeding in the fresh unheated state. The residue of the serum was invariably filtered through Berkefeld V filters and kept in sterile ampoules in the cold. Such filtered sera retained their agglutinin-content for long periods and those with highest agglutinin-content, whether for myxoma or fibroma or both, proved extremely useful as positive controls in subsequent work with fresh samples.

The actual micro-agglutination test.—The writer has had a long experience of this method of performing agglutination tests in hanging-drop preparations of elementary body suspensions. Its great merit is that it economises precious material, tediously won, which can serve for lengthy periods, and there is no evidence that the results of such tests and the inferences based upon them are any less authoritative than those derived from macro-methods, which demand a considerable sacrifice of animals and the manipulation of large masses of infective material.

The upper surfaces of the rings sealed to the slides should be ground smooth and level, and should take comfortably a coverslip of 1 inch square. The ringed slides are prepared by pressing lightly on vaseline heated on a copper plate. Coverslips (new) are taken from the box, rubbed lightly with a silk rag, passed through the Bunsen flame several times rapidly, and laid on a black tile.

The tube containing the elementary body suspension, after removal from the cold room, is lightly rotated to disperse any elementary body masses deposited by gravity and then spun for a few minutes in the water turbine centrifuge. This precaution should never be neglected, as it ensures the removal not only of spontaneously agglutinated masses of elementary bodies, which are sometimes apt to form in suspensions kept for longish periods, but also of particles of fluff, etc., which gain entrance to the opened tube into which the finely drawn-out pipette with fiduciary mark is dipped. With this tube the necessary quantities of suspension are delivered to the slips of a titration series, after which, by aid of the same pipette, the serum dilutions prepared from the centrifuged serum sample are incorporated with the suspension, beginning with the weakest. The ringed slides are then carefully pressed down on the slips and rapidly inverted. Proper sealing is secured by pressing the portion of slip resting on the vaselined ring with a wooden rod. The slides are then marked and placed on a wooden bench at room temperature with a dust cover. Readings may be made from the second or third day onwards, a definitive reading being registered on the sixth or seventh day. A two-thirds inch objective, with substage condenser removed, is adequate, but it is an advantage to be able to turn on the sixth-inch objective, particularly in the later stages of the reaction, when the products of agglutination or of simple deposition have settled to the bottom of the drop. Just as in completed macro-tests with bacteria in round-bottomed tubes, a reading may be made from the appearance of the mass at the bottom of the unshaken tube, so in the hanging-drop preparation a similar preliminary reading can be made from the disposition of the matter lying on the bottom curvature of the drop. It is advisable, however, to agitate the drop by careful rocking of the slide, particularly in the first 48 hours, as this greatly facilitates rapidity of clumping. It has been said that the micro-method may give trouble from casual development of bacterial contaminations or moulds in the drops during the course of incubation. This has been the rarest of occurrences in the writer's experience.

EXPERIMENTAL RESULTS.

Preliminary Experiments with casually taken Sera from Myxoma and Fibroma Animals.

(A) Five rabbits bled on October 19th, 1935, representing intervals of 2 months (*a*); 15 months (*b*); 16 months (*c*); 3 months (*d*); and 12 months (*e*) after recovery from myxoma infection naturally contracted (experiments of C. J. M.). These five sera were tested with *M.E.B.* (*i. e.* myxoma elementary body suspension) on December 17th, 1935, along with sera from two fibroma-infected rabbits and a normal rabbit.

Result.

Rabbit *a*: 8++++, 16+, 32+, 64 tr.
Rabbit *b*: 8+, 16+, 32 tr.
Rabbit *c*: 8+, 16 tr.
Rabbit *d*: 8 tr.
Rabbit *e*: 8 tr.
Fibroma (12th day): 8++++, 16+.
Fibroma rabbit (18th day): 8, 0; 16, 0.
Normal rabbit: 8, 0; 16, 0.

Remarks.—These early results indicated clearly that agglutinins for myxoma were demonstrable not only in animals recovered from myxoma naturally contracted, but also in fibroma-infected rabbits, whereas normal rabbit serum was devoid of such in the highest concentration used. An affinity between the two viruses in serological behaviour was thus already apparent.

Tests with Fibroma Elementary Body Suspension (*F.E.B.*)

A suspension of *F.E.B.* became available for use on December 24th, 1935, and occasion was taken to retest two of the sera which had reacted most powerfully with *M.E.B.*, viz. rabbit myxoma (*a*) and rabbit fibroma (12th day).

Only one concentration of serum was used (1 in 8) and it was found that both sera agglutinated *M.E.B.* and *F.E.B.* completely in this dilution. Titration to end-point of serum of rabbit fibroma (12th day) carried out frequently in subsequent months showed a steady maintenance of agglutinin-titre both for *M.E.B.* and for *F.E.B.* in the filtered sample kept in cold storage. Thus on May 20th, 1936, a complete titration of this serum and of that from another fibroma rabbit (1415 A) bled by Dr. Hurst three days after intradermal inoculation in four areas with 10 p.c. fibroma virus, yielded the following result:

Fibroma (12th day)	*v.M.E.B.*:	8++++, 16++++, 32+++, 64+, 128 tr.
	v.F.E.B.:	8++++, 16++++, 32+++, 64++, 128 tr.
1415 A (3rd day)	*v.M.E.B.*:	8+++, 16+++, 32+, 64+.
	v.F.E.B.:	8+++, 16+, 32 tr.

Results with two other casual sera from fibroma-infected animals may be added, viz., 1414 A (9th day) and 1412 A (7th day). The former agglulutinated *M.E.B.* in 1 in 64 and the latter in 64–128. Neither agglutinated *F.E.B.* in the highest concentration used.

In the case of Rabbit 1415 A the rapid development of agglutinins for *M.E.B.* in such a short period as three days is in keeping with and probably goes far to explain the very early onset of resistance to myxoma exhibited by fibroma-infected animals.

The information from tests of these casual samples of sera from fibroma-infected animals and from animals which had recovered from myxoma contracted by contact in rabbit compounds, raised hopes that the development of agglutinins for the infecting virus would be normally accompanied by a *pari passu* development of agglutinins for the allied virus. As we shall see, however, from results of the inquiry now to be detailed into the course taken by the agglutinins in individual rabbits studied over long periods, agglutinin development for *M.E.B.* was the chief feature of the response both to myxoma and to fibroma infection, while significant titres for *F.E.B.* were not invariably recorded. In the experiments which follow, the end-points only of the titrations will be entered.

Course of Agglutinins in Rabbit "NR".

This rabbit was inoculated on December 14th, 1935, on one side of shaved back (R) with descending concentrations to 1 in 1000 of fibroma virus mixed with equal parts of normal rabbit serum and on the other (L) with similar concentrations of virus mixed with pooled antifibroma serum from two rabbits killed on 12th and 18th days respectively after infection with fibroma virus. On the 6th day the diameters of the fibroma domes on the right side, representing concentrations of 10^{-0}, 10^{-1}, 10^{-2}, 10^{-3}, were 20, 10, 7 and 3 mm., whereas all doses on the left side were completely neutralized.

Tests of bleedings.—Before inoculation: *v.M.E.B.* 4–8, *v.F.E.B.* 4. 6th day: *v.M.E.B.* 8–16, *v.F.E.B.* 4. 12th day: *v.M.E.B.* 16–32, *v.F.E.B.* 4–8. 19th day: *v.M.E.B.* 128, *v.F.E.B.* 4. 26th day: *v.M.E.B.* 128, *v.F.E.B.* 4.

On February 10th, 1936, when the myxoma titre was still 64 and the fibroma titre remained at 4, the animal was re-inoculated in two I.Ds. with fibroma and gave an immune response. Tests on the 2nd and 5th days after this re-inoculation yielded myxoma titres of 32–64 and 16–32 respectively, but no change in the fibroma titre.

On February 22nd, 1936, when the myxoma titre was 8–16 and the fibroma titre 4, the animal was inoculated with myxoma in two I.Ds. In response, an accelerated skin-reaction resulted, elevated pink thickenings appearing by the 2nd day and becoming necrotic in centre on the 4th day. Very slow regression. On 20th day skin-lesions almost flat with black central sloughs. No eye discharge or other evidence of general invasion.

Tests of bleedings yielded on 6th day *v.M.E.B.* 256, *v.F.E.B.* 128; on 11th day *v.M.E.B.* 16, *v.F.E.B.* 4–8; and on 19th day *v.M.E.B.* 4, *v.F.E.B.* 4.

After an interval of two months the animal received a second inoculation with myxoma on May 16th, 1936, in two I.Ds.

Result.—Slightly elevated pink nodes on 2nd day, which were shrinking and scaling on 6th day. On 10th day still hard shotty nodes, which disappeared only slowly. Tests of sera on the 3rd and 6th days after the re-inoculation showed a rise of the myxoma titre to 32, but no change in the fibroma titre. After an interval of nine months the animal received a third inoculation of myxoma on February 26th, 1937, in 4 I.Ds. The allergic skin response was on this occasion considerable, large elevations with dusky purple centres being

recorded on the 5th day. The elevations became confluent by the 11th day. There were no eye discharge or other signs of generalization. Again the serum tests showed only slight elevation of the myxoma titre.

Remarks on Rabbit " NR ".—The primary inoculation of this animal with fibroma led to a considerable development of agglutinins for myxoma, but no appreciable development of agglutinins for the infecting virus. Re-inoculation with fibroma had little effect on the myxoma agglutinins and did not stimulate the formation of agglutinins for fibroma. When, however, the animal received for the first time an injection of myxoma virus, the effect on the agglutinins was striking, a very high titre of agglutinins both for myxoma and for fibroma being recorded on the 6th day. This accelerated response was, however, of short duration. Two further inoculations of myxoma in the course of subsequent months appear to have exerted only a slight stimulating effect on the residual myxoma agglutinins at least within the 10-day periods following inoculation.

Further Tests of Animals Infected with Shope Virus in its Fibromatous and " Inflammatory " Varieties.

Rabbit (2) received Shope virus (fibroma strain) in 6 I.Ds. on October 19th, 1936, and Rabbits (4) and (5) received similar inoculations of the " inflammatory " strain on the same date.

Results of test bleedings :

Rabbit (2).			Rabbit (4).			Rabbit (5).	
Day.	*v.M.E.B.*	*v.F.E.B.*	Day.	*v.M.E.B.*	*v.F.E.B.*	Day.	*v.M.E.B.*
Before inoculation	8	<4	Before inoculation	<4	4	Before inoculation	4
5th	4	<4	5th	32	8–16	5th	64
10th	64	<4	10th	128	32	10th	128
15th	8	<4	15th	64	16	18th	256
19th	<4	8	19th	4	8	27th	64–128
						33rd	16

It will be noted that Rabbit (4), after inoculation with the inflammatory strain of the Shope virus, exhibited a high development of agglutinins both for myxoma and fibroma, a phenomenon not invariably observed in animals receiving the ordinary " fibromatous " strain. The high and early development of agglutinins for myxoma by Rabbits (4) and (5) is also notable. One example was met of a fibroma-infected animal (fibromatous strain) which failed to produce significant agglutinins even for myxoma during the first 20 days. After an interval of 8 months, however, this animal was given a dose of myxoma which, as in the case of Rabbit " NR ", had the effect of inducing the formation of both myxoma and fibroma agglutinins in high titre (256 for myxoma and 64 for fibroma on the 12th day).

Agglutinin Development after Infection with (A) *Virulent Myxoma Virus and* (B) *with the Neuromyxoma Variant.*

(A) *Virulent myxoma infection.*—It was not to be expected that during the short course of a myxoma infection ending in death generally well within ten days, any very significant development of agglutinins would occur. On the

other hand, the fact that animals experimentally infected with myxoma remain active, and, in spite of possibly extensive local lesions, eat well till about the 6th or 7th day, when they relapse into complete apathy, refuse food, and exhibit signs of general invasion, made it at least possible that stimulation of the antibody-forming mechanism might in some degree take place. This proved to be so and one example is given here in which a normal rabbit received 2 I.Ds. of myxoma (Aragão strain) on January 16th, 1936.

Before inoculation : *v.M.E.B.* 4 tr., *v.F.E.B.*<4. 2nd day : *v.M.E.B.* 4, *v.F.E.B.* 4. 4th day : *v.M.E.B.* 8–16, *v.F.E.B.* 4. 6th day : *v.M.E.B.* 16–32, *v.F.E.B.* 4–8. 9th day : *v.M.E.B.* 32, *v.F.E.B.* 4–8.

The 9th day sample was taken while the animal was moribund. Eye discharge had been noted first on the 7th day. It will be seen that the animal developed not only a fair titre of agglutinins for *M.E.B.*, but also a significant amount of agglutinin for *F.E.B.* In this respect its behaviour recalled that of the animal infected with the inflammatory strain of the Shope virus.

(B) *Neuromyxoma infection.*—The first animal to be tested was inoculated in 5 I.Ds. with neuromyxoma brain material and it was proposed to take blood samples on the 5th and 10th days and at later periods. The virus, however, proved unexpectedly lethal and the animal was found dead on the 9th day. It had shown some slight eye discharge on the 8th day. The dose admittedly was large and the local skin reactions were massive. Before inoculation on October 19th, 1936, the serum gave the following : *v.M.E.B.* 4 tr, *v.F.E.B.* 4 tr., but on the 5th day the reading was *v.M.E.B.* 64, *v.F.E.B.* 4 tr., showing a considerable production of agglutinins for myxoma in this short time.

The second animal received 4 I.Ds. of neuromyxoma brain material on October 29th, 1936. Severe local reactions ensued, and on the 9th day localized myxomatous nodes appeared on the eyelids, but without conjunctival discharge. On the 12th day some discharge was noted from nose and eyes, but it was fairly evident that the animal had passed the critical stage. It made a satisfactory recovery in spite of obvious generalization of virus manifested by nodes in eyelids and satellite nodes on the shaved back.

Serum Tests (*v.M.E.B. only*).

Dates of bleeding.	*v.M.E.B.*
Before inoculation	4
5th day	4
9th day	64
14th day	16–32
22nd day	8
37th day	8–16

On January 30th, 1937, the serum failed to agglutinate either *M.E.B.* or *F.E.B.* in concentration of 1 in 4. On February 26th, it received 4 I.Ds. of virulent myxoma, which produced extensive granulomatous lesions, but did not lead to a fatal issue. The agglutinin titre responded to the new infection by rising on the 5th and 11th days to 16 and 32 respectively.

The Reponse of Myxoma-Recovered Animals (Contact Infections) to Fresh Doses of Myxoma or Fibroma Virus.

Three of these animals, viz. " b " and " d ", which were bled and tested on October 19th, 1935 (see above), and " g ", which had its primary attack of myxoma in August, 1934, became available for study on January 10th, 1936.

Rabbit " d ", which had recovered from naturally contracted myxoma in August, 1935, was given, on January 16th, 1936, 2 I.Ds. of myxoma virus (Aragão strain). An intense accelerated skin reaction of hæmorrhagic type resulted, which only very slowly cleared up. The animal made a good recovery in the course of the following four weeks.

Serum Tests.

Dates of bleeding.	*v.M.E.B.*	*v.F.E.B.*
Before inoculation	4	<4
2nd day	64	<4
4th day	128	16–32
6th day	32–16	<4
9th day	64	4
12th day	32	4

On March 10th, 1936, this rabbit, whose skin-lesions had by now entirely disappeared, was given another dose of myxoma in 2 I.Ds., a similar dose being administered to a normal control, which succumbed in standard time. The skin reaction on this occasion was minimal, only two small hardish nodes appearing, which declined without showing any central necrosis. This result was of some interest as showing that an active immunity to this virus could be conferred of such degree as to keep in abeyance even the usual skin response to the virus. This second dose adminstered to rabbit " d " produced a rise of myxoma agglutinins from <4 to 16 on the 4th day.

Rabbit " G ".—This rabbit, now in the 18th month of convalescence from naturally contracted myxoma, was given on February 10th, 1936, a dose of fibroma in 2 I.Ds. of a testicular sample. Only a trivial skin reaction followed. Before inoculation this animal, in spite of the long period of convalescence, was found to have still a high titre of agglutinins (64) for myxoma, though none were demonstrated for fibroma in 1 in 4. On the 2nd day the titre rose to 128 *v.M.E.B.*, but on the 5th day it had fallen to 8. Agglutinins for *F.E.B.* did not appear in titratable amount.

Agglutinin-Absorption Experiments.

In order to secure more precise information with regard to the relationship of fibroma virus antigen to that of myxoma—more particularly desirable in view of the Berry-Dedrick experiment—it had been planned to attempt absorption experiments on recognized lines provided the large concentrations of purified elementary bodies necessary for such work on an extended scale became available. As we have already indicated, this proviso will probably be best secured in future by technical operations permitting the manipulation of much larger quantities of infective material than those adequate for micro-agglutination work. However, it was decided to explore in a preliminary way

the possibilities of an absorption technique adapted to micro-methods. Two experiments on these lines were carried out and as the results were fairly clean-cut, they are here given in some detail.

The sera tested were two, viz. *A*, from a rabbit on the 12th day after infection with fibroma virus, and *B* from a rabbit on the 6th day after inoculation with myxoma virus superposed during recovery from a primary fibroma infection. Both sera had been filtered and stored in the cold. For absorption fairly thick suspensions of myxoma and fibroma were prepared. 1 c.c. of these suspensions was mixed with 1 c.c. of the " immune " serum diluted 1 in 2 and placed in the hot room at 37° C. for one and a quarter hours. The contents of the tubes were then centrifuged at 15,000 r.p.m. and the supernatants carefully removed. These were again centrifuged water-clear in the water centrifuge and tested for residual agglutinin content by the usual micro-methods with the stock suspensions employed for this purpose.

Results.

	Serum dilutions.	Serum A.	Serum B.
Before absorption, *v.M.E.B.*	8	++++	+++
	16	++++	++++
	32	+++	++
	64	+	+
	128	tr.	0
,, ,, *v.F.E.B.*	8	++++	++++
	16	+++	+++
	32	++++	++
	64	++	0
	128	tr.	0
After absorption with *M.E.B.*			
v.M.E.B.	8	0	tr.
	16	+	±
	32	++	tr.
	64	±	0
,, ,, *v.F.E.B.*	8	0	0
	16	0	0
	32	0	0
	64	0	0
After absorption with *F.E.B.*			
,, ,, *v.M.E.B.*	8	tr.	0
	16	+	0
	32	+	tr.
	64	tr.	0
,, ,, *v.F.E.B.*	8	0	0
	16	0	0
	32	0	0
	64	0	0

In both experiments the results indicated that absorption of the sera either with *M.E.B.* or *F.E.B.* removed completely the original content of fibroma

agglutinins. On the other hand, the effect of the absorptions on the myxoma agglutinins was to leave some slight but definite traces of agglutinin spread over several dilutions. There was almost a suggestion that absorption with fibroma virus was somewhat more effective in removing the last traces of myxoma agglutinins than the myxoma suspension itself. Reliable deductions as to the precise relationship of the two viruses are not possible on the basis of these absorption experiments. They serve at the most to emphasize the close affinity between them, as disclosed by the data recorded in this communication. To meet the criticism that absorption of these not very high-titred sera might effect a mere mechanical removal of agglutinin, my colleague, Dr. Schütze, added the same absorbents to two agglutinin-containing sera, one *v.B. para A* and the other *v.B. typhosus*. In neither case was the agglutinin-titre disturbed.

DISCUSSION.

Shope (1932) in the course of his search for virus agents that might possibly be kin to the fibroma virus accidentally discovered by him in the cottontail, made the important observation that domestic rabbits, which had recovered from fibroma as they invariably do—for fibroma is a benign infection—do not as a rule succumb when subsequently inoculated with myxoma virus, an agent which almost invariably kills the domestic rabbit in 10 days or less. What is the nature of this relationship between two viruses otherwise so dissimilar both clinically and pathologically ?

Towards the solution of this problem Shope, in his first paper and in subsequent communications (1936*a*, *b*) has contributed experimental data based mainly on virus-neutralization tests and infection experiments, while Hurst (1937*c*) has added more precise particulars regarding the time factors in the development of this resistance to myxoma exhibited by fibroma-recovered rabbits. It should be clearly understood that this resistance is manifested essentially by its power to keep in abeyance an otherwise certain fatal issue. The capacity to produce local lesions at the site of intradermal inoculation may persist and may be accompanied by the appearance of myxomatous nodes in other regions of the body. It is, therefore, as a rule of the nature of a partial immunity, but one sufficient to avert death.

The ascertained facts may be briefly stated. The rabbit which has recovered from a fibroma infection, is immune to a later dose of the same virus, and its serum, while neutralizing readily the fibroma virus, has, so far as Shope's experiments indicated, no neutralizing action on the myxoma virus. If the fibroma-recovered animal is subsequently inoculated with myxoma virus, it shows itself highly resistant, and, though local lesions may ensue, it makes, as a rule, a good recovery. The serum of such animals has then marked neutralizing action both on myxoma and on fibroma virus. Rabbits which recover, as they may, if extremely rarely, from a myxoma infection, are resistant to fibroma and their sera neutralize fibroma as well as myxoma virus. With regard to the time factor, Shope (1936*b*) found that neutralizing bodies for myxoma could not be demonstrated in the serum of fibroma-recovered rabbits subsequently inoculated with myxoma virus, before the 7th day. Resistance to myxoma, however, could be demonstrated in Hurst's (1937*c*) experiments

as early as the 2nd day after the introduction of the fibroma virus at which time the superposed inoculation of myxoma was made. To these observations, from which Shope very reasonably inferred that the antigen of the fibroma virus was only a fraction of the full myxoma virus antigen and that the two conditions were most probably distinct, must now be added the findings of Berry and Dedrick (1936), in great part confirmed by Hurst (1937*c*), which would seem to postulate an actual transformation of fibroma to myxoma virus. If these mixtures of heated myxoma and active fibroma virus are inoculated into fibroma-immune animals the " transformation " does not take place, so that the process would seem to depend on the biological activity of the fibroma virus in the test animal, a condition on which, as Shope early pointed out, the development of resistance to myxoma exhibited by the fibroma-recovered animal also seemed to rest. Hurst (1937*c*) has added the information that the neuromyxoma variant, whether in the heated or in the " living " state, is incapable of assisting the transformation of fibroma to myxoma. We must await further analysis of the mechanism of this remarkable experiment before accepting the inference that a real transformation of fibroma virus to myxoma virus takes place, even though the conditions under which it is claimed to occur admittedly resemble those which determine a change of type in the Griffith experiment with pneumococci. What light do the serological data here presented throw on the nature of the relationship? In the first place, as the writer reported in 1936, it is clear that the fibroma-infected rabbit produces with fair regularity agglutinins for myxoma in the course of the fibroma infection. With less regularity are agglutinins produced for the homologous fibroma virus except in the case of animals infected with the " inflammatory " strain of this virus. The fibroma virus, therefore, can induce the formation of " group " agglutinins for myxoma in greater concentration than it can the formation of homologous agglutinins. What is the significance of this in view of the fact that the serum of the recovered fibroma animal has been shown to exert little or no neutralizing action on myxoma virus and yet the animal is highly resistant to this latter virus?

Such resistance has been noted, as we have said, in the very early days after inoculation of fibroma and high agglutinin-contents for myxoma virus have also been observed at this early period. It would seem reasonable to assume that these agglutinins play some part in the mechanism of the resistance exhibited towards a superposed myxoma infection. It is recognized that specific agglutinins can play *in vivo* an important part in localizing infective bacteria, and I suggest that here also the presence in the blood of the fibroma animal of agglutinins for myxoma virus may have the very salutary effect of converting an otherwise fatal disease to one in which the tissue responses to myxoma virus, both local and general, assume the more localized and nodular type as opposed to the diffuse invasive lesions ending inevitably in death. An analysis of my own experiments in which myxoma was given, without fatal issue, to fibroma-recovered animals clearly reveals this change in the type of lesion. The development of myxoma agglutinins in response to fibroma receives a further stimulus of the accelerated type when myxoma virus is subsequently introduced, and this further rise is often accompanied by a simultaneous appearance of agglutinins in high titre for fibroma, though such latter

agglutinins may not have revealed themselves in the course of the primary fibroma infection.

That the fibroma rabbit develops agglutinins for myxoma elementary bodies has been also the experience of Rivers and Ward (1937), who have succeeded in obtaining by centrifugal methods a sufficiency of the pure washed elementary bodies of myxoma to serve for macroscopic agglutination tests. Fibroma-recovered animals subsequently infected with myxoma and hyperimmunized with the same virus over a period of two months yielded sera which agglutinated myxoma in a concentration of 1 in 256, while sera from recovered fibroma animals agglutinated the same suspensions of myxoma in 1 in 64. Re-inoculations of myxoma after the first do not appear in the writer's experience to be very effective in raising to high values a titre which has been allowed to drop to low levels, but I have not tested the effect of hyperimmunization carried out on the scale and with the time intervals employed by Rivers and Ward. In any case the experiments detailed in this communication show that fibroma animals and particularly those receiving the "inflammatory" strain develop in the course of infection high agglutinin titres for myxoma equalling those resulting from hyperimmunization.

Though myxoma is almost invariably fatal, the infected animal is capable of developing agglutinins of moderate titre both for myxoma and for fibroma. So also does the animal infected with the neuromyxoma variant and the animal which succeeds in recovering from myxoma contracted by contact with infection in the warren.

To summarize the main results, it is clear that both viruses, when independently inoculated produce agglutinins not only for the homologous but also for the cognate virus. The fibroma animal, in fact, produces agglutinins more readily for myxoma than for the homologous fibroma. The agglutinin-absorption experiments, admittedly tentative and incomplete, would at least support the view that the two antigens, in so far as the fractions responsible for production of agglutinins for myxoma or fibroma are concerned, are extremely closely related, possibly identical. Taken, however, in conjunction with the known facts relating to the virus-neutralizing bodies, the most reasonable assumption would seem to be that the myxoma antigen alone contains a fraction capable of stimulating the formation of neutralizing bodies for myxomal while both contain fractions capable of inducing agglutinins and neutralizing bodies for fibroma, together with agglutinins for myxoma. On this assumption the fibroma mosaic would differ from that of myxoma solely by its lack of a fraction capable of inducing the formation of neutralizing bodies for myxoma. Whether the Berry-Dedrick experiment signifies the acquisition of this latter fraction by the fibroma antigen when the virus is growing *in vivo* in contact with heat-inactivated myxoma antigen, must be left for further inquiry.

The resistance or partial immunity to myxoma exhibited by the fibroma-recovered animal would depend in the writer's view on the localizing action exerted by already circulating myxoma agglutinins on the invading myxoma virus. This localizing action in the case of a myxoma infection superposed at an early date after infection with fibroma would also possibly be aided by the non-specific inhibitory effect due to simultaneous proliferation of another virus.

ADDENDUM: ON THE IMMEDIATE CAUSE OF DEATH IN MYXOMATOSIS OF RABBITS.

This has not so far been adequately elucidated by pathological and histological examination of fatal cases on which there is now a considerable literature. Death has often been quite sudden in my experience and in that of Dr. Hurst. A rabbit, while in the late apathetic stage and refusing all food, has been seen to fall over dead, though a few minutes before it may have strongly resisted handling for purposes of a bleeding. The slow and stertorous respiration in the later stages has not found in many cases any really adequate explanation from autopsy findings in trachea and lung, though this latter organ is frequently the site of lesions of varying inportance and intensity, a descriptive summary of which has been given by Hurst (1937*a*). I would regard the most important pulmonary change that, when present, may well contribute to the fatal issue, to be the alveolar hæmorrhages and the large bull's eye hæmorrhages that may beset the surface of the lung like an experimental purpura. In a later paper I propose to discuss the nature of the tissue responses to myxoma in the light of findings that have emerged from histological examination of material accumulated in the course of this work. Here I would simply note that in the search for the more immediate cause of death my attention has been concentrated on the bone-marrow, an organ whose examination at autopsy has hitherto, strangely enough, been neglected, and that the presence there of extensive cytotoxic change in cells of the myelocyte and erythrocyte series with accompanying profuse hæmorrhages, may well be the main contributory cause of death and sudden death in rabbit myxomatosis, particularly when taken in conjunction with the alterations in the blood picture to which Hobbs (1928) first drew attention.

CONCLUSIONS.

(1) Rabbits infected with fibroma virus develop agglutinins in their sera which act both on myxoma and on fibroma.

(2) On the localizing action *in vivo* of such myxoma agglutinins probably depends the resistance exhibited by fibroma-recovered animals to superposed infection with myxoma.

(3) Rabbits which recover, as they may do, if rarely, from a myxoma infection naturally contracted, and rabbits infected with the neuromyxoma variant of Hurst, contain in their sera agglutinins both for myxoma and for fibroma.

(4) The fibroma and myxoma viruses are probably not identical when serologically analysed, the former being distinguished by its lack of an antigen fraction capable of stimulating the formation of myxoma-neutralizing antibodies.

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THE ALLEGED ANTITOXIC ACTION OF VITAMIN C IN DIPHTHERIA.

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THE isolation and chemical identification of vitamin C (*l*-ascorbic acid) was followed by an indiscriminate search for possible functions of this interesting compound other than its antiscorbutic activity. Although evidence obtained before this period did not promise well, the bearing of vitamin C on various immunological reactions has also been receiving its ample share of attention during the last few years.

In some early experiments (Zilva, 1919) it was found that the serum of guinea-pigs suffering from chronic scurvy after immunization with *B. typhosus* yielded agglutination and amboceptor titres of the same order as that obtained from the serum of normally-fed animals similarly immunized. Nor did the chronic vitamin C deficiency influence the activity of the complement of sera from scorbutic guinea-pigs. In the case of diphtheria (Arkwright and Zilva, 1924 ; Bieling, 1925) it was observed that after intra- or subcutaneous injections of diphtheria toxin or living bacilli in guinea-pigs suffering from chronic scurvy, the inflammatory reaction was decidedly less than when the animals used were kept on a normal and well-balanced diet. That this was not due to the vitamin C deficiency *per se* was evident from the fact that animals subsisting on a quantitatively restricted diet which contained ample quantities of vitamin C showed similarly an attenuated local reaction. Bieling had observed at the same time that scorbutic guinea-pigs succumbed to diphtheria toxin more readily than normally-fed animals, but the disease was so far advanced in his animals at the time of injection that beaded costo-chondral junctions were found at autopsy, and consequently the lowered resistance to the action of the toxin cannot be justifiably ascribed to a specific vitamin C deficiency.

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The work of Harde and her collaborators (Harde, 1934; Harde and Philippe, 1934; Greenwald and Harde, 1935) was the first sign of returning interest in the subject of the influence of vitamin C on the susceptibility of the animal organism to diphtheria toxin. Impressed by the fact that in guinea-pigs which received diphtheria toxin, the adrenals suffered a diminution in their vitamin C content, whilst those of the mouse, which synthesizes the vitamin, and which was found to be resistant to 1–10 M.L.D. of diphtheria toxin, retained their full load of ascorbic acid, even after the injection of the toxin, they instituted experiments with the object of studying the action of ascorbic acid on diphtheria toxin *in vitro* and *in vivo*. In the former case it was observed that the addition of neutralized ascorbic acid had a destructive effect on diphtheria toxin, and although no clear-cut data were obtained *in vivo*, the results were considered by these workers to favour the view that vitamin C had a protective action against diphtheria toxin. In the same connection King and Menten (1935) found that when guinea-pigs were partially depleted of their vitamin C body-reserve without showing signs of scurvy, and injected with graduated doses of diphtheria toxin, their survival time was shortened by 50 p.c., and their loss in body-weight was greater than in the case of animals with higher stores of vitamin C. Jungeblut and Zwemer (1935) also concluded from their experiments *in vitro* and *in vivo* that vitamin C plays an important part in the mechanism of natural resistance to diphtheria toxin. It is of interest to note that these workers, unlike Arkwright and Zilva and Bieling, found that the storage of vitamin C by the guinea-pig had a pronounced effect in inhibiting or even suppressing the local reaction after intracutaneous injections of diphtheria toxin. Jeney, Gagyi and Baranyai (1936), too, claimed that a daily dose of 1–2 mg. of ascorbic acid imparted higher resistance to guinea-pigs against diphtheria toxin. The observations of Bock and Grossmann (1936) also lent support to the view of the antitoxic property of vitamin C in diphtheria. They instilled *C. diphtheriæ* into the conjunctiva and found that the eye reaction was least marked in those animals which received an excess of ascorbic acid, and most marked in scorbutic guinea-pigs.

Although a number of workers claim to have obtained beneficial results in human as well as in guinea-pig diphtheria, by using a mixture of the cortical extract of the adrenals and ascorbic acid (used apparently because of the involvement of the suprarenal glands in diphtheria) (Herbrand, 1935; Bamberger and Zell, 1936; Thaddea, 1937; Schmidt, 1937; and others), the results obtained in the case of human beings with ascorbic acid alone were not promising. Thus Widenbauer and Saretz (1936) still found a positive Schick reaction in patients after administering a high dose of ascorbic acid (1400 mg.) or daily doses of the vitamin (50 mg.) for prolonged periods; yet the intracutaneous injection of a mixture of 0·2 mg. of diphtheria toxin and 50 mg. of ascorbic acid into a Schick-positive patient yielded a negative result. Otto (1936) observed no difference in the condition between two groups of patients who received 500 units of antitoxin per kg. of body-weight with or without the addition of 500–700 mg. of ascorbic acid, in spite of a retention of the vitamin by the patients of the former group.

The belief entertained by some that ascorbic acid possesses antitoxic properties in diphtheria is, therefore, based on the three following main

observations: (*a*) that in guinea-pigs suffering from diphtheria there is a marked diminution in the ascorbic acid content of the adrenals, (*b*) that ascorbic acid destroys the activity of diphtheria toxin *in vitro*, and (*c*) that guinea-pigs receiving generous quantities of vitamin C are more resistant to the effect of diphtheria toxin than animals which are deficient in ascorbic acid. The first two reasons can be dismissed as unconvincing, since the depletion of the adrenals and *of other organs* of vitamin C is not specific to diphtheria, but may be traced to various causes, and is in all probability secondary in nature. Nor is the destructive action of ascorbic acid on diphtheria toxin *in vitro* a proof that the vitamin possesses antitoxic activity; many substances inactivate diphtheria toxin *in vitro* without being able to exercise antitoxic action *in vivo*. Whether the deleterious effect of ascorbic acid on diphtheria toxin *in vitro* is due to its acidity, as maintained by some workers, or to its reducing property as maintained by others, is irrelevant to the present issue. If, however, ascorbic acid can, as it is claimed, modify the action of diphtheria toxin *in vivo*, the problem becomes of great interest. In the experiments to be described this claim could unfortunately not be substantiated.

TECHNICAL.

The guinea-pigs, which belonged to a vigorous stock, were kept during the pre-experimental period on a diet of oats, bran and fresh cabbage *ad lib.* About 40–50 g. per day of the latter, which is roughly equivalent to 40–50 mg. of ascorbic acid, was consumed by each guinea-pig. All the animals were vaccinated with a mixture of *Salmonella enteritidis* (Gaertner) and *S. typhi murium* (Aertrycke), and only those which were making very good growth were used. The diphtheria toxin, for which I am indebted to Dr. G. F. Petrie, of the Lister Institute, was injected subcutaneously in the abdominal region. The ascorbic acid doses, which were dissolved and partially neutralized immediately before use, were injected into the femoral muscle. When the injection of the vitamin was continued for several days the doses were introduced into the femoral and biceps muscles in rotation in order to avoid local discomfort. In the case of the guinea-pigs which received the pre-experimental mixed diet or extra quantities of ascorbic acid, the oats, bran and cabbage *ad lib.* were continued. During the first day after the injection the animal usually consumed almost as much cabbage as during the pre-experimental period, but as the disease developed the daily consumption of the cabbage diminished; it, however, never fell below an equivalent of 5–10 mg. of ascorbic acid. The guinea-pigs which were depleted of vitamin C were kept for 5 or 6 days before injection on a scorbutic diet which consisted of bran 6 parts, barley meal 2 parts, middlings 3 parts, fish meal 1 part, crushed oats 4 parts by volume, and no more than 60 ml. of autoclaved milk made up from a dried powder. This diet was in their case continued to the end of the experiment.

RESULTS.

No full appreciation of the results can be obtained until the general response of the guinea-pig organism to the administration of various quantities of vitamin C is brought into focus. When these animals are maintained on a

mixed diet such as the above they become " saturated " with the vitamin, that is to say the ascorbic acid content of the tissues reaches a concentration which cannot be raised by a further increase in the oral consumption of vitamin C. In this condition certain tissues, such as the adrenal (about 1·5 mg./g.), the small intestine (about 0·15 mg./g.), liver (about 0·25 mg./g.), contain much more than the bulk of the tissue such as the muscle and bone (about 0·04 mg./g.). The blood-plasma content is in this case on the average only about 0·5 mg. per 100 ml., if, which is probably the case, its very slight indophenol-reducing capacity be taken to be due to the presence of ascorbic acid. The condition of " saturation " persists only above a certain level of intake of vitamin C. Below this level the tissue content of ascorbic acid shows a tendency to diminish, and may even almost disappear in animals which are receiving daily doses as high as 5–6 times the minimum dose necessary to protect them from scurvy. Another striking feature is that the body-reserve of vitamin C of a fully " saturated " guinea-pig practically disappears after the animal is maintained on a scorbutic diet for only five or six days. Animals so depleted of vitamin C are then found to be in excellent health, to grow normally, and to be free from scurvy ; no ascorbic acid can, of course, be detected in their plasma. Although the vitamin C content of the plasma even of " saturated " guinea-pigs is very low, this can, nevertheless, be raised considerably at will, by injecting large doses of ascorbic acid. During the first few hours after injection high quantities of the injected vitamin are circulating in the blood, in spite of the tendency of the animal to excrete this excess quickly in the urine. The following representative experiment will illustrate the point : A series of guinea-pigs of about 250 g. in weight which were previously kept on a mixed diet with cabbage *ad lib.* were injected with 50 mg. of *l*-ascorbic acid. Animals were killed ½ hour, 1 hour, 2 hours, 2½ hours and 4 hours after the injection, and the ascorbic acid was determined in the plasma. The figures obtained were 32·5 mg., 17·5 mg., 11 mg., 7·5 mg. and 3·5 mg. per 100 ml. of plasma respectively.

The results of all the experiments are summarized in the table. The data have not been arranged chronologically but, for convenience, in ascending order of the quantities of vitamin C consumed by the various groups of guinea-pigs. Even a cursory glance at the figures makes it clear that the resistance of the animals to the diphtheria toxin was the same and independent of the level of intake of vitamin C. In experiments D and E (see table) one group of the animals was depleted of vitamin C and consequently their tissues, including the blood, contained very little, if any, ascorbic acid. On the other hand, the other group which was on diet containing cabbage *ad lib.*, *i. e.* consuming *per os* an equivalent of about 10–40 mg. of ascorbic acid per day, received in addition 250 mg. of ascorbic acid by injection twice daily for 2½ days after the introduction of the toxin. The figures given above will indicate the very high concentrations of ascorbic acid in the plasma which were reached under these circumstances. Such concentrations could not be attained from the diet, however rich in vitamin C, even if it be assumed that under conditions of stress all the ascorbic acid contained in the tissues is thrown into the circulation. In spite of this abnormally high ascorbic acid content of the plasma the injected animals did not show any greater resistance to the toxin than did the

Expt.	Diet.	Mg. *l*-ascorbic acid injected.	M.L.D.	Weight (g.). Initial.	Weight (g.). Final.	Survived days.
D	Vitamin C-depleted for 5 days on scorbutic diet	None	1	325	272	3
			1	305	285	2–3
			1	330	262	4
			1	330	315	3
			1	350	255	10*
			1	310	265	10*
E	Vitamin C-depleted for 6 days on scorbutic diet	,,	1	225	202	4–5
			1	255	220	11
			1	250	200	11*
			1	242	185	6–7
			1	240	170	11*
			1	245	202	4–5
B	Mixed diet	,,	1	250	217	3–4
			1	245	200	4–5
			1	255	255	2
C	,,	,,	1·5	250	265	1–2
			1·5	255	250	3
			1·5	257	255	2–3
A	,,	,,	2	260	257	1–2
			2	255	237	2
A	Mixed diet	50 About 10 mins. before injection of diphtheria toxin	2	250	250	1–2
			2	255	240	1–2
			2	255	240	2
			2	252	257	1
B	,,	75 Just before injection of diphtheria toxin	1	250	225	3–4
			1	253	262	2–3
			1	245	205	4–5
C	,,	,,	1·5	250	235	3–4
			1·5	250	240	2
			1·5	250	227	2
D	Mixed diet	250 in 50-mg. doses twice daily, morning and afternoon. First dose injected 15 mins. after diphtheria toxin	1	310	325	2
			1	325	272	4
			1	305	285	4–5
			1	330	305	5–6
			1	330	305	3–4
			1	350	285	10*
E	,,	,,	1	250	245	9*
			1	250	225	4–5
			1	247	205	4
			1	242	195	9*
			1	252	215	9*
			1	242	200	5–6

* Killed by chloroform.

guinea-pigs which were depleted of the vitamin. It is, therefore, not surprising that the animals on the mixed diet with or without single injections of ascorbic acid reacted similarly. These results, which are clear-cut, do not support in any way the contention that vitamin C possesses antitoxic properties against diphtheria toxin.

SUMMARY.

Experiments are described which do not support the view that vitamin C has a protective action against diphtheria toxin in guinea-pigs.

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FURTHER STUDIES OF THE AGENT OF THE ROUS FOWL SARCOMA: A. ULTRA-CENTRIFUGATION EXPERIMENTS; B. EXPERIMENTS WITH THE LIPOID FRACTION.

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A. ULTRA-CENTRIFUGATION EXPERIMENTS.

THE technique of adsorption and elution with aluminium hydroxide was originally designed by Wilstätter for the isolation of enzymes. It was first used by Fraenkel (1929) for the isolation of the agent of the Rous fowl sarcoma and its successful use in this way was believed to prove the enzymatic nature of the agent. As, however, the same method was subsequently used for the

isolation of certain other viruses of a truly contagious nature, this argument cannot stand. The optimum conditions for the method of adsorption and elution of the agent of the Rous fowl sarcoma have been studied and published by us (1935). The majority of the workers in this country are, however, of the opinion that the agent is a micro-organism similar to those which are the cause of variola and other contagious diseases. Evidence exists, however, in support of the opinion that such viruses vary greatly among themselves. Fraenkel (1931) placed some of these viruses, such as the bacteriophage, the tobacco mosaic virus and the Rous fowl sarcoma, in a special group under the name " virus enzymes ", consisting of agents which are not of a particulate nature, but more probably active molecules in colloidal solution which, as in the case of the tobacco mosaic virus, might be obtainable in a pure, crystalline form.

Ledingham and Gye (1935) and Amies (1936 and 1937) are of the opinion that " elementary bodies " obtained by a process of fractional ultra-centrifugation of tumour extracts are the actual virus of the Rous fowl sarcoma, and much evidence has been collected in support of that view. We have performed a number of experiments involving ultra-centrifugation and have compared the minimal amount of extract, supernatant and deposit necessary for tumour production. The results of ten experiments are shown in Table I. In four of them the concentration of the agent was increased in the sediment in relation to the original extract. In three the original extract and in two even the supernatant were shown to possess a greater degree of activity than the deposit. We have also endeavoured to compare the number of " elementary bodies " present with the power of tumour production of the material in which they were observed (Table II). The experiments showed no relation between an estimate of the number of " elementary bodies " present and the minimum dose necessary to produce a tumour. The experiments were carried out with both centrifugates and eluates, and it is especially clear from the examination of eluates which contain very few " elementary bodies " that there is no proportional relationship between the number of these " elementary bodies " and the power of tumour production of the solution. For these reasons it seems probable to us that in the case of the deposit being rich in both " elementary bodies " and the agent the latter is adsorbed to the " elementary bodies ". There is, however, still the possibility that the activity is associated with certain " elementary bodies " which are alone present in the eluates and which form a very small proportion of the large number of " elementary bodies " of an inert nature which are present in the sediment (Amies, 1937).

From aqueous extracts of the Rous sarcoma Claude (1937*a*) obtained a sediment by means of a similar technique of fractional ultra-centrifugation. This sediment contains the bulk of the agent and has an isoelectric point at pH 3·8 ; it is inactive at a lower pH and is also inactivated at pH 12. It has an absorption band in the ultra-violet end of the spectrum at 2575 Å. which corresponds closely to Pentimalli's (1936) findings. Pentimalli showed by means of diffusion experiments the presence of a characteristic protein containing phosphorus, which is either the agent or the carrier of the agent and which also has an absorption band in the ultra-violet at about 2800 Å. The diffusion liquid from the tumour-bearing wing of fowls with the Rous fowl sarcoma contained about 0·7 mg. per 100 c.c. of this protein.

TABLE I.—*Minimal Dose in c.c. necessary for the Production of Tumour of Fractions of Rous Fowl Sarcoma Extract produced by Centrifugation at 15,000 r.p.m.*

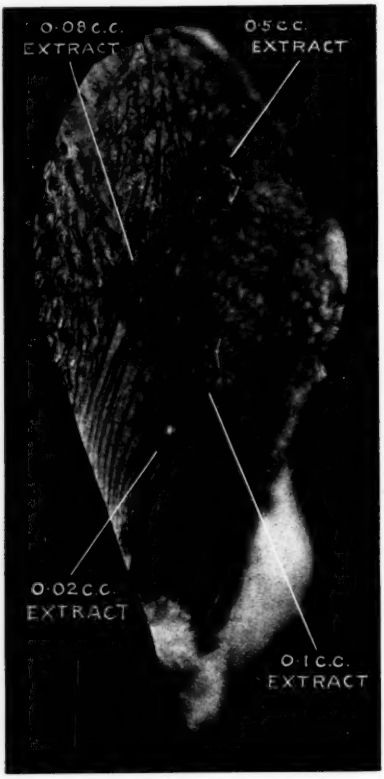
Date of experiment.	Extract.	Supernatant.	Deposit.	
4.ii.36	0·04	0·024	0·06	..
18.ii.36	0·1	0·5	0·3	..
2.iii.36	0·01	Died	0·003	X
5.iii.36	0·1	3·0	0·01	X
24.iii.36	1·0	5·0	0·3	X
2.iv.36	0·001	5·0	0·3	..
1.v.36	0·01	0·005	0·05	..
21.v.36	0·005	0·5	0·2	..
6.viii.36	2·0	Died	0·08	X

The sign "X" in the last column indicates an experiment in which a tumour was produced by a quantity of sediment smaller than the smallest quantity of the corresponding extract that was necessary to produce a tumour.

TABLE II.—*Comparison of the Number of Elementary Bodies and the Power of Tumour Production.*

No.	Material injected.	Estimate of elementary bodies.	Minimal dose producing tumour in c.c.
1	Extract[1]	++	0·04
	Supernatant[2]	+	0·024
	Deposit[3]	++++	0·06
2	Extract[1]	+++	0·005
	Supernatant[2]	+	0·5
	Deposit[3]	+++	0·2
	Second supernatant[4]	+	2·0
3*a*	Extract[1]	++++	No tumour
	Supernatant from adsorption[5]	+	0·08
	Deposit from adsorption supernatant[6]	++++	No tumour
3*b*	Eluate[7]	+	No tumour
	Deposit from eluate[8]	++	2·0
	Supernatant from eluate[9]	+	0·2

[1] Extract: Ringer extract of tumour tissue.
[2] Supernatant: Supernatant remaining after the centrifugation of extract at 15,000 r.p.m. for 2 hours.
[3] Deposit: Deposit obtained from the above treatment of extract.
[4] Second supernatant: Supernatant remaining after the deposit had been suspended in saline and re-centrifuged at 15,000 r.p.m.
[5] Supernatant from adsorption: Supernatant remaining after the alumina had been deposited by centrifugation at 3500 r.p.m. in the course of the adsorption.
[6] Deposit from adsorption supernatant: The deposit obtained from the above material when centrifuged at 15,000 r.p.m.
[7] Eluate: Material obtained by the elution of the alumina referred to in 5 with phosphate buffer.
[8] Deposit from eluate: Deposit obtained by the centrifugation of eluate at 15,000 r.p.m.
[9] Supernatant from eluate: Supernatant remaining after the operation in 8.



Fraenkel and Mawson.

In a recent communication (1936) we pointed out that the process of fractional ultra-centrifugation, would, in itself, be likely to produce a sediment composed of corpuscles of uniform physical qualities and size, and this opinion was confirmed when we carried out the fractional ultra-centrifugation of extracts of normal tissue and of glycogen solution, which process resulted in the production of " elementary bodies " of the same appearance. The fact that eluates, containing very few " elementary bodies ", are still very active and rich in agent excludes the idea of the agent being essentially represented by the " elementary bodies ".

Evidence afforded by serological observations has recently been subject to doubt. The fact already observed by Rous, that antibody formation becomes weaker as the agent used becomes more pure shows that the antibodies are not caused by the agent itself, and this has, in our opinion, been confirmed by the neutralizing effect of anti-fowl sera on purified tumour agent suspensions (Amies, 1937). Des Ligneris (1934) explains the weak action of anti-Rous sera by the demonstration of an inhibitory substance in the fowl serum. All the evidence afforded in proof of the particulate and therefore extrinsic nature of the virus is, for all these reasons, not sufficiently strong.

B. EXPERIMENTS WITH THE LIPOID FRACTION.

An experiment in which varying quantities of the Rous sarcoma extract were injected into the skin of a bird gave further evidence in support of our opinion of the chemical nature of the agent. The photograph shows the skin of a chicken injected with different quantities of an extract of Rous fowl sarcoma tissue. The quantities in question were 0·5 c.c., 0·1 c.c. 0·08 c.c., 0·02 c.c. and 0·01 c.c. The times of appearance of the tumours were 11 days, 11 days, 13 days and 18 days respectively. Only four tumours are to be seen as the last of these amounts did not give rise to one. The time of the first appearance and the ultimate size of any particular tumour depend entirely upon the amount of extract injected. It is apparent to us, that such large differences as may be observed, produced by quantities of extract the order of difference in size of which is so small, indicate the existence of an agent of a chemical nature rather than that of a particulate micro-organism.

A paper published by Jobling and Sproul (1936) seemed to settle the question in favour of the chemical theory. They claimed that repeated injection of acetone extracts of fresh Rous sarcoma tissue produced similar tumours. They further stated that a second acetone extract of the residue would produce tumours only if incubated with extracts of Kieselguhr granulomata from the breast-muscle of normal fowls. Carbon tetrachloride extracts of dried and powdered Rous sarcoma tissue were also able to produce tumours if incubated with the same tissue. We have tried to repeat this work in ten experiments, but have so far obtained completely negative results.

In our own experiments, when extracts of fresh tissue were prepared these were centrifuged at a speed of 3500 r.p.m. and were filtered through paper before the solvent was removed. The injections were made over a long period of time and the instructions of Jobling and Sproul were followed as closely as possible.

Pollard and Amies (1937) have come to the conclusion that tumours produced by the lipoid material extracted from the dried tissue of Rous fowl sarcoma No. 1 are due to the contamination of the lipoid with traces of the tumour desiccate. When the lipoid was completely freed from particulate matter by filtration it had no carcinogenic properties. These findings are in complete agreement with our own, as we have always filtered our extracts before using them. This does not necessarily mean that the agent itself is of the nature of a virus, but merely that it was contained in the fraction insoluble in acetone and carbon tetrachloride.

In two recent papers by Jobling, Sproul and Stevens (1937*a*, *b*) results are given together with a complete description of their methods. In the first paper the technique they describe is similar to that which we have employed, but the authors remark that it is not offered as a method worthy of repetition, as the results obtained were far more reliable when the technique described in the second paper was used. In the second paper they report that they have been able to isolate the agent in a highly purified lipoid extract of vacuum-desiccated material. The nitrogen content of these extracts was 0·36 p.c. of the total solids extracted and the reducing substances amounted to 0·22 p.c. The lipoid is, itself, inactive, but can be activated by ascorbic acid, extracts of normal muscle and their protein fractions, by casein, gelatine and gum acacia.

Technique of the preparation of powdered dry Rous sarcoma tissue.—The fresh tissue was minced n a fine tissue mincer and placed in a large mortar surrounded by a freezing mixture. The tissue was then pulped vigorously with a pestle until a stiff, frozen, froth-like mass was obtained. This mass was transferred to a shallow dish and placed in a desiccator over 100 p.c. sulphuric acid. The desiccator was then evacuated, refilled with nitrogen from a cylinder and re-evacuated. The tissue was completely dry within a few hours. The dry material was powdered in a mortar.

Technique of the extraction of dry Rous sarcoma tissue with acetone and carbon tetrachloride.—The technique of the extraction was the same for each solvent. The powdered dry tissue was shaken in a small bottle together with the solvent for a period of one hour, a mechanical shaker being used. The extract was centrifuged, filtered and evaporated at 37° C. under reduced pressure and the residue after evaporation redissolved in a small quantity of acetone. The solution thus obtained was poured into Ringer solution and the acetone removed by a stream of nitrogen. Mixtures of extract and residue emulsion or of extract and granuloma tissue were incubated together for one hour at 37° C. The whole of the operations were carried out in an atmosphere of nitrogen.

In the course of our experiments we made a few observations which have led to entirely different conclusions to those of Jobling and Sproul. We found that the injection of the residue remaining after the extraction of the dried and powdered Rous sarcoma tissue with acetone and carbon tetrachloride produced tumours. In these experiments neither the extracts themselves. nor mixtures of the residues with the extracts resulted in the growth of tumours when injected. Our experiments show that the agent cannot be extracted by means of acetone and carbon tetrachloride. This does not, however, exclude the conception of the agent as a " virus enzyme ", a substance which, like other enzymes, might be assumed to be insoluble in these liquids.

The Experiments 1–3, described below, show that the residue remained active while mixtures of the residue with the extract were inactive ; it thus seems possible that an inhibitor was present in these extracts :

(1) An experiment was carried out on November 3rd, 1936, in which a specimen of powdered dry Rous sarcoma No. 1 tissue, obtained from the Rockefeller Institute for Medical Research in New York, was extracted with acetone. The powder was approximately 18 months old. Injection of the extract alone and the extract plus the residue into Chick No. 379 did not produce a tumour whereas the injection of the residue alone produced a tumour after 30 days.

(2) Another experiment was carried out with the same material on November 25th, 1936, and positive results were obtained with the residue in Chick No. 393 after 28 days, while the injection of the extract alone and mixtures of extract and residue were without result.

(3) A third experiment was carried out with a specimen of powder produced in this laboratory which was approximately 6 months old. This experiment was performed on February 19th, 1937, and in this case the extraction was carried out with carbon tetrachloride. Injection of the residue alone produced a tumour in 17 days, while the injection of the extract alone or mixtures of the extract and residue was without result.

We propose to attempt the isolation of inhibitors by means of this method, which may be of considerable importance in the problem of their chemical identification. So far inhibitors have been obtained by the method of Jobling and Sittenfield (1931) in which the agent is precipitated at pH 4 together with inert protein, while the inhibitor remains in solution together with a number of inert substances. Murphy and Claude (1933), using a greater concentration of alumina, obtained the inhibitor in the adsorbate. Murphy (1936) also uses extracts heated to 56° C. for 30 minutes as an inhibitor.

SUMMARY.

1. Concentration of the active agent of the Rous fowl sarcoma is not invariably obtained in the deposit after fractional centrifugation for 2-hour periods at 15,000 r.p.m.

2. There is no relation between the tumour-producing activity of a preparation and the number of " elementary bodies " visible therein.

3. Small variation in the quantity of Rous fowl sarcoma extract injected produces tumours different in size and time of appearance in the same bird, pointing to the chemical nature of the agent.

4. No agent could be obtained by means of extraction with acetone and carbon tetrachloride.

5. These extracts seemed to contain an inhibitor, whereas the agent may be obtained from the residue remaining after extraction of the dried Rous fowl sarcoma tissue.

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THE PREPARATION AND COMPARISON OF DIFFERENT TYPES OF ANTI-TYPHOID SERA. (AGGLUTININS—MOUSE PROTECTION AND PRELIMINARY CLINICAL TRIALS.)

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IN publications which appeared in 1927 and 1928, a description was given by Grasset and Gory of a method of preparing a therapeutic antityphoid serum, using for the hyper-immunization of the horses a formolized derivative of the endotoxin of *B. typhosus*, termed " endotoxoid " by Grasset in later investigations on this new type of antigen (1935). This serum, which possesses both antibacterial and antitoxic properties, has been prepared and improved upon by this last author (Grasset, 1931, 1933) at the South African Institute for Medical Research during the past nine years. It has been used during the same period in the treatment of more than 3500 cases of typhoid fever with therapeutic success, resulting in a reduction of more than 50 p.c. in the mortality-rate in the series of patients treated (Grasset, 1930, 1931).

Felix and Pitt (1934) discovered the existence of the Vi antigen of *B. typhosus*, and prepared an anti-typhoid serum active against this antigen by injection of the Vi strain. We then set out to make a comparative study of the preparation and the specific properties of the different sera obtained by the above two methods, and also of serum prepared by combining the two methods.

PREPARATION OF THE DIFFERENT TYPES OF ANTI-TYPHOID SERA.

The following types of anti-typhoid sera were prepared in the Serum Department of the Institute, and their respective properties compared :

1. Serum obtained from typhoid endotoxoid.
2. Serum obtained from live virulent Vi *B. typhosus* strains.

3. Serum obtained from live avirulent Vi *B. typhosus* strains.

4. Serum obtained from the combined immunization by typhoid endotoxoid and Vi virulent strains.

5. Serum obtained by mixture *in vitro* of serum 1 and serum 2.

TECHNIQUE OF IMMUNIZATION.

Serum 1 (endotoxoid).—A new horse, No. 252, was immunized according to the procedure hitherto used in the department for anti-typhoid serum production. It was submitted to increasing amounts of formolized typhoid endotoxoid obtained from *B. typhosus* strains selected on account of their high antigenic power, *i. e.* high toxicity and pathogenicity to mice and tested for O inagglutinability and virulence by the methods laid down by Felix for the detection of Vi antigen; these strains proved to be also rich in this antigen. Immunization commenced on March 26th, 1936, and consisted of 10 subcutaneous injections at 8–10-day intervals, of respectively 10, 20, 40, 60, 80, 100, 150, 200, 250 and 300 c.c. of the antigen. The horse was bled 8 days after the last injection.

Serum 2.—A. Horse No. 254, also new, was immunized according to the method of Felix with increasing concentration of emulsions of live virulent Vi strains, Ty 2 and Watson, which were received from the Lister Institute, through the courtesy of Dr. Felix. The immunization was carried out from March 5th to May 14th, and consisted of 9 weekly intravenous injections of live emulsion of 24-hour culture on ascitic agar. The injections were all given in a suspension of 20 c.c., the concentration increasing progressively from 500 millions to 5000 millions per c.c.

B. Horse No. 253 was immunized according to the same method, but two *B. typhosus* strains of South African origin were used as antigen, namely, strains Ty 5 and 7753/6, the latter isolated two months previously. These two strains, which were specially investigated among other locally isolated strains with regard to their antigenic properties, proved experimentally to be as rich in Vi antigen as the two Vi strains, Ty 2 and Watson, mentioned above (Grasset and Lewin, 1936). It is interesting to note that strain Ty 5 was selected in 1932 by one of us (E. G.) for the preparation of typhoid endotoxoid, prior to any knowledge of the Vi antigen, in consequence of its high pathogenic, endotoxic and immunizing properties. It has been kept since on ordinary agar, and used during these four years for the preparation of this antigen.

Serum 3.—The hyper-immunization of horse No. 275 was carried out with two avirulent Vi variant strains, Ty 441R5 and 6S, rich in Vi antigen, but poor in "O", which we were fortunate to receive also from Dr. Felix. The same technique of immunization was used as with horses Nos. 253 and 254. The series of 9 injections was spread over a period of about two months.

Serum 4.—Horse No. 252, which, as mentioned above, was originally immunized with formolized endotoxoid (Serum 1), was submitted to a re-immunization by a combined inoculation of formolized endotoxoid and intravenous injection of live Vi virulent strains of Ty 2, Watson, Ty 5 and 7753/6. The two types of antigen were given alternately, at weekly intervals, over a period of three months, from July to October, 1936.

Serum 5.—This was obtained by mixture *in vitro* of equal parts of Serum 1 (endotoxoid) and Serum 2 (live virulent Vi strain).

AGGLUTININ CONTENT OF SERA.

The determination of the specific agglutinin content of H, O and Vi was carried out for the various sera. The H agglutination was performed with a formolized suspension of strain H 901 as antigen; for the O agglutination an alcoholic extract of strain O 901 was used, according to the technique already employed by us in previous investigations (Grasset and Lewin, 1936). The Vi agglutination was performed according to the technique of Felix, by absorbing the sera with H 901 at 37° C. for 4 hours, centrifuging, and using for the agglutination test the supernatant (*i. e.*, absorbed diluted serum) with a saline suspension of live Ty 2 emulsion, obtained from a 24-hour ascitic agar growth. The mixtures were incubated at 37° C. in a water-bath for 2 hours, and then

left at room temperature for a further 22 hours, when they were examined. All readings were taken macroscopically with a lens in daylight.

The results of these tests are shown in Table I.

TABLE I.—*Agglutination Titres of the Various Sera.*

Serum.	H.	O.	Vi.	
1. Endotoxoid serum	100,000	51,200	—	—
2. Anti-Vi serum—				
*(*a*) Ty 2, Watson	12,800	25,600	800	1,600‡
† (*b*) Ty 5, 7753/6	100,000	25,600	1,600	3,200‡
3. Anti-Vi serum—				
* (*a*) Avirulent 441R5, S6	6,400	200	800	3,200‡
4. Combined immunization — endotoxoid Vi strain	100,000	25,600	800	3,200‡

* Lister Institute strains. † South African strains. ‡ Titres of Vi agglutinins corresponding to sera obtained after a second immunization four months later.

From Table I it will be noticed that all the sera contained H agglutinins, and all, with the exception of Serum 3, contained O agglutinins to comparatively high titre. Serum 3, made from avirulent Vi strains, contained only a very small amount of O agglutinins. It is questionable whether differences in agglutinin titres obtained by different methods can be altogether ascribed to differences in the power of the antigens used for the immunization, as variation in the reaction of the individual horses must be considered. Although several horses have been inoculated for each of the different types of sera, during these last two years, it was unfortunately not possible to extend these comparative investigations to a greater number of sera, on account of the large number of mice necessary for these tests. However, the fact that endotoxoid Serum 1, although possessing high O and H agglutinins, contains no Vi antibodies, may be ascribed to the absence of this antigen in the vaccine, as serological investigations show that persons inoculated with typhoid endotoxoid vaccine do not develop this antibody, in spite of H and O agglutinins to high titre (Grasset and Lewin, 1936.)

It is known that rough strains of *B. typhosus* contain very little, if any, smooth O antigen, and this would account for the small amount of O agglutinin in Serum 3, *i. e.* from the horse inoculated with the avirulent rough Vi strains. The sera obtained from the horses inoculated with live Vi strains all contained H, O and Vi agglutinins, as was to be expected.

In our series we thus consider that the agglutinins found in the various types of sera are in accordance with those expected, regard being paid to the type of antigen used in the immunization.

PROTECTION AGAINST VI ANTIGEN AFFORDED BY THE DIFFERENT TYPES OF SERA TO MICE.

These tests were carried out on white mice of 22 to 25 g., and of homogeneous breeding. Strain Ty 2 was selected for all the tests, and emulsions of 24-hour

growth on ascitic agar were used. In all the cases the emulsion of living micro-organisms was injected intraperitoneally, care being taken to verify the stability and virulence of the strain in a preliminary test. Each of these tests was accompanied by a series of tests on control mice to ascertain the fatal dose for the actual test.

Different modes of experimentation were tried. Preliminary investigations showed us that the technique consisting in the injection of the virulent emulsion and of the serum after contact *in vitro* leads to irregular and fallacious results; consequently this method was discarded. In most of the tests we followed the technique used in previous experiments by Grasset (1931), *i. e.* subcutaneous injection of the serum followed 18 hours later by intraperitoneal injection of the typhoid culture. In some other tests the serum was injected 18 hours before the typhoid emulsion, but intramuscularly, according to the technique used in some experiments by Felix. These methods were both found to be satisfactory. Mice used in these tests were kept under observation during the 48 hours following the injection of the typhoid culture, and results were recorded at the end of this time. Four to six mice were used for each dose; in large comparative experiments this number had, however, to be reduced to three.

The object of the first experiment was to determine which of the sera afforded protection to the Vi antigen of Felix, and the relative degree of the protection. Mice were inoculated subcutaneously with 1 c.c. of the respective sera, and 18 hours later varying doses of live typhoid organisms were injected intraperitoneally, representing three, five and seven fatal doses of this strain. The results of this experiment are given in Table II:

TABLE II.—*Protection Tests with Mice against* B. typhosus, *Ty 2, 18 hours after Injection of 1 c.c. of Serum subcutaneously.*

Serum.	Proportion of survivors.		
	Three fatal doses.	Five fatal doses.	Seven fatal doses.
Serum 1 (endotoxoid) . . .	2/3	1/3	0/3
,, 2*a* (Vi strain, virulent) . .	3/3	2/3	0/3
,, 3 (Vi strain, avirulent) . .	3/3	3/3	1/3
,, 4 (Combined immunization endotoxoid, Vi strain) .	3/3	3/3	1/3
,, 5 (Mixed serum, endotoxoid and Vi strain) . .	3/3	3/3	3/3

Control mice (unprotected) 1 fatal dose, 100 millions, 0/12 survived.

It will be seen that all the sera showed some degree of protection against the live strains of *B. typhosus* Ty 2, and it is particularly important to note that Serum 1 (endotoxoid), which contains no Vi agglutinins, afforded some protection. This result does not accord with the findings of other workers (Felix and co-workers, 1934–1935), for whom Vi antibodies are of primary importance, if not essential, in the defence against attack by similar strains of *B. typhosus*, though some antibacterial activity of the O antibody can be demonstrated in phagocytic experiments with strains of high virulence (Felix

and Bhatnagar, 1935). According to these authors, O antibody is mainly responsible for effecting the neutralization of the endotoxin of *B. typhosus*; that the other sera, which all contain Vi antibodies, afford protection is to be expected.

Serum 3, prepared from avirulent Vi strain, and containing very little O antibody, showed a very good degree of protection against live Ty 2. It thus appears that the presence of a large amount of O antibodies in the serum is not necessary for the protection against live Vi organisms. These findings agree with those of Felix and Pitt (1935).

In order to determine the limit of protection of Serum 1 (endotoxoid) against Ty 2, the following complementary experiment was carried out. Mice injected subcutaneously with 1 c.c. of Serum 1 were tested intraperitoneally 18 hours later with one, two and three fatal doses of Ty 2 respectively, *i. e.* 100, 200 and 300 millions of this strain.

The results in Table III show a partial protection against three fatal doses, as in the previous experiment, but complete protection is afforded against one and two fatal doses of this strain.

TABLE III.—*Protection afforded to Mice by Serum 1 (Endotoxoid) against One, Two and Three Fatal Doses of Strain Ty 2.*

Vi strain Ty 2.	Proportion of survivors.		
	One fatal dose.	Two fatal doses.	Three fatal doses.
Serum 1. 1 c.c. (endotoxoid) . .	6/6 .	6/6 .	5/6

Control mice (unprotected) 1 fatal dose, Ty 2, 100 millions, 0/6 survived.

Another comparative experiment was carried out to estimate more closely the relative protecting power of the endotoxoid serum and Vi-containing sera. Mice were injected intramuscularly with 0·2 and 0·4 c.c. of sera types 1, 2*a* and 4, and tested 18 hours later with three fatal doses of live Ty 2 intraperitoneally.

The tabulated results (Table IV) show that whilst the highest degree of protection is conferred by Vi Serum 2*a*, an appreciable protection by Serum 1 (endotoxoid) is again exerted against live Vi organisms.

TABLE IV.—*Protection afforded to Mice by Serum 1, 2a and 4, 0·4 and 0·2 c.c. by the Intraperitoneal Injection of Three Fatal Doses of* B. typhosus *Ty 2, 18 Hours later.*

Amount of serum injected.	Proportion of survivors.		
	Serum 1 (endotoxoid).	Serum 2*a* (Vi strain).	Serum 4 (combined endotoxoid and Vi strain immunization).
0·4 c.c. . . .	4/6 .	6/6 .	5/6
0·2 c.c. . . .	3/6 .	6/6 .	4/6

Control mice (unprotected) 1 fatal dose, 100 million, Ty 2, 0/6 survived.

This observation is in agreement with the experiments of Ørskov and Kauffmann (1936), on the prophylactic action of endotoxoid serum against

limited amounts of Vi typhoid bacilli. These authors note that when small doses of Vi typhoid bacilli are injected intraperitoneally into the mouse, the Lister Serum containing Vi antibodies can eliminate the typhoid bacilli very effectively, but that an O serum (South African Institute) is nearly as potent.

From the results of comparative protection tests incorporated in Table II it is shown that the highest protection is exerted by Serum 5, obtained by the mixture *in vitro* of Serum 1 (endotoxoid) and Serum 2*a* (Vi strains) in equal parts. In order to determine the limit of protection exerted by this mixed serum, series of mice received 1 c.c. of Serum 5, subcutaneously, and were tested 18 hours later with an increasing number of fatal doses of strain Ty 2 injected intraperitoneally. The respective fatal doses were 3, 5, 7, 9, 10, 12 and 15. The results are in Table V, and show that full protection is manifested against 9 fatal doses of *B. typhosus* Ty 2 (900 millions), whilst partial protection is still observed up to 15 fatal doses.

TABLE V.—*Protection afforded to Mice by 1 c.c. of Serum 5, Mixed Endotoxoid Serum + Anti-Vi Serum against Increasing Numbers of Fatal Doses of Ty 2.*

Fatal doses of *B. typhosus* Ty 2.	Proportion of survivors.						
	3.	5.	7.	9.	10.	12.	15.
Serum 5 1 c.c.	3/3	3/3	3/3	3/3	2/3	1/3	1/3
,, 5 0·5 c.c.	3/3	3/3	1/3	—	—	—	—

Control mice (unprotected), 1 fatal dose, 100 millions, *B. typhosus* Ty 2, 0/6 survived.

Repeated experiments conducted on the same lines afforded similar results. In view of these results, protection tests were done with the same serum (Serum 5), but with injections of 0·5 c.c. instead of 1 c.c. The number of fatal doses of Ty 2 used in this test was limited to 3, 5 and 7. All the mice submitted to the inoculation of 3 and 5 fatal doses survived the test (Table V).

The results recorded in the last two experiments show that the protection conferred by this mixed serum not only compares favourably with, but notably exceeds the degree of protection observed with the two constituent sera (*i. e.* Vi anti-serum and endotoxoid O serum) which entered into its mixture, and also of that of the other types of sera submitted to these comparative investigations.

PROTECTION AFFORDED TO MICE BY THE DIFFERENT TYPES OF ANTI-TYPHOID SERA AGAINST O ANTIGEN.

The previous experiments showed that a serum rich in Vi, but poor in O antibodies, gave a solid protection against live Vi organisms (Serum 3, avirulent Vi strains), and that a serum rich in O, but devoid of Vi antibody, gave a lesser degree of protection (Serum 1, endotoxoid). In the following experiments it was sought to determine whether a serum rich in Vi, but poor in O antibody, would protect against live O antigen. Accordingly, mice were injected subcutaneously with 1 c.c. of the different types of anti-typhoid sera, and 18 hours later live typhoid organisms (strain O 901) were inoculated intraperitoneally. The results of the experiment are given in Table VI.

TABLE VI.—*Protection afforded to Mice by 1 c.c. of Various Sera Injected Subcutaneously against Two and Three Fatal Doses of* B. typhosus *O 901 Injected Intraperitoneally 18 Hours later.*

Type of serum.	Proportion of survivors. 2 fatal doses O 901.	3 fatal doses O 901.
Serum 1 (endotoxoid) . . .	5/6	5/6
,, 2*a* (Vi strain, virulent) . .	6/6	4/6
,, 3 (Vi strain, avirulent) . .	1/6	0/6
,, 4 (combined immunization endotoxoid, Vi strain) .	6/6	6/6
,, 5 (mixed serum, endotoxoid and Vi strain) .	6/6	6/6

Control mice (unprotected), 1 fatal dose, 500 millions, 2/12 survived.

It will be seen that all the sera which contain O antibody afforded a degree of protection against live O antigen. The only serum which did not confer much protection is Serum 3 (avirulent strain), which, though rich in Vi antibody, is very poor in O antibody. The results relating to Serum 1 and 3 are particularly interesting for comparison, inasmuch as the former does not possess any Vi antibody, whilst the latter possesses smooth O antibodies in negligible quantity only. We consider that the findings observed with regard to the other sera are not of much importance for comparison, as all contain both O and Vi antibodies to relatively high titre.

In order to compare more closely the relative anti-O properties and protection conferred by the different types of sera 1, 2*a*, 2*b* and 3, injections of these sera in amounts of 0·4 c.c. and 0·6 c.c. were given to mice intramuscularly, followed 18 hours later by two fatal doses of live *B. typhosus* O 901. The results are given in Table VII.

TABLE VII.—*Protection afforded to Mice by 0·4 c.c. and 0·6 c.c. of Different Types of Anti-typhoid Sera Injected Intramuscularly against Two Fatal Doses of* B. typhosus *O 901, injected Intraperitoneally 18 hours later.*

Types of serum.	Proportion of survivors. 0·4 c.c. I.M.	0·6 c.c. I.M.
Serum 1 (Endotoxoid) . . .	3/6	5/6
,, 2*a* (Vi strain)	2/6	2/6
,, 2*b* (Vi strain)	3/6	2/6
,, 3 (Vi strain, avirulent) . .	0/6	0/6

Control mice (unprotected), 1 fatal dose, 500 millions, 0/6 survived.

From this experiment it will be seen that the highest degree of protection against O antigen is afforded by Serum 1 (endotoxoid), which also possesses the highest O agglutinins; a similar action, but to a lesser degree, is exerted by Sera 2*a* and 2*b*, obtained from live virulent Vi strains, whilst no protection is observed with Serum 3, which is almost devoid of O antibodies, having been derived from the horse with live avirulent Vi strains.

EXPERIMENTS RELATIVE TO ANTITOXIC NEUTRALIZATION OF TYPHOID ENDOTOXIN.

Neutralization experiments carried out on the antitoxic powers of Serum 1 (endotoxoid), against the typhoid endotoxin, show that 1 c.c. of this anti-O serum is able to neutralize *in vitro*, after one hour contact, 5 fatal doses of *B. typhosus* endotoxin obtained from Vi or O typhoid strains ; above 5 fatal doses neutralization becomes irregular. Typhoid endotoxins used in these neutralization tests were obtained by five operations of alternate freezing and thawing of concentrated emulsions of *B. typhosus* submitted to extraction either alive or after heating at 56° C., the soluble endotoxin being finally separated from the bacterial residue by centrifugation. A similar degree of antitoxic action has also been observed with the same serum in neutralization tests, performed by mixing serum with increasing fatal doses of intact bacterial emulsions of *B. typhosus*, heated for 45 minutes at 58° C.

We shall note, finally, a point of interest in the phenomenon of interaction of typhoid endotoxin serum mixtures. As recorded in a previous communication (Grasset, 1934), by mixing *in vitro*, fixed amounts of typhoid endotoxin and increasing volumes of typhoid serum, one or sometimes two zones of specific flocculation can be observed in the series of tubes, according to the sera or strains used in the tests. Recent investigations on this subject, with the different types of typhoid sera under investigation, have shown that whilst one zone of flocculation is observed with Serum 1, *i. e.* anti-O serum, mixed with endotoxin of Vi strains, two zones of flocculation are observed when this Vi endotoxin is mixed with the Vi antisera, Serum 2*a*, Serum 2*b* and Serum 3. Experiments are proceeding with a view to studying more closely the relationship of these zones of flocculation to the corresponding antibodies contained in these sera.

PRELIMINARY COMPARATIVE CLINICAL INVESTIGATION WITH ENDOTOXOID ANTI-O SERUM (SERUM 1) AND VI ANTI-SERA (SERUM 2A AND 2B).

Whilst serological investigations were still in progress, opportunity was taken of the last typhoid season to carry out a comparative clinical trial with Serum 1 (endotoxoid anti-O serum) and a mixture of Serum 2*a* and 2*b*, anti-Vi sera, respectively. From November, 1936, to March, 1937, twenty typhoid patients at the Johannesburg Hospital, bacteriologically or serologically confirmed as such, were treated ; alternate patients receiving concentrated " endotoxoid " serum and anti-Vi serum, both of which have approximately the same O titre (1/50,000), the anti-Vi content of the letter serum being 1/1600. Volumes of serum in both series varied from 80 to 100 c.c., given intramuscularly in daily doses of 20 c.c. In most of the cases serum treatment was instituted during the second week of the infection ; in a few, during the first week.

We will not enter into the details of these early comparative investigations, which are to be incorporated, after completion, in a later publication ; we may say, however, that in the first series no marked difference could be noticed in

the intrinsic therapeutic action of the two types of sera on the evolution of the infection. Whilst undoubtedly both types of sera exerted beneficial action on the evolution of the disease, as described in previous papers dealing with the clinical use of the respective anti-typhoid sera (Grasset, 1930 ; Felix, 1934), no great difference could be observed between them. At most the detoxicating action appeared to be stronger in some of the severe toxic cases treated with " endotoxoid " serum.

In view of the experimental findings described above, and the indication of the optimum protection conferred by the mixed serum (anti-endotoxoid O serum, and anti-Vi serum), a new series of comparative treatments will be instituted to test clinically the therapeutic action of this last type of serum.

CONCLUSIONS.

From the series of comparative investigations on the different types of anti-typhoid sera, the following facts may be elicited :

Protection against Vi B. typhosus.

1. An anti-typhoid serum rich in Vi antibody will protect against invasion of live Vi *B. typhosus*, whether the serum contains O antibody in addition or not.
2. An anti-typhoid serum rich in O antibody, but devoid of Vi antibody, will also confer a protection against invasion of Vi organisms, but to a lesser degree than a serum rich in Vi antibody.

Protection against " O " B. typhosus.

1. An anti-typhoid serum rich in O antibody, but devoid of Vi antibodies, will afford solid protection against invasion by live O organisms.
2. An anti-typhoid serum rich in Vi antibody, but devoid of O antibody, will afford no protection against invasion with live O organisms.

In our investigations maximum protection of mice has been observed with a serum obtained by mixture of high titre anti-O serum from horses immunized with formolized endotoxoid, and of a high titre anti-Vi serum from horses submitted to live emulsions of Vi typhoid bacilli. This serum proved, in our hands, to confer the highest protection against the invasion of both O and Vi typhoid bacilli.

From these considerations, therefore, it appears that such an anti-typhoid serum containing a maximum of both O and Vi antibodies would prove the most efficacious in treatment of typhoid cases, whatever may be the antigenic nature of *B. typhosus* responsible for the typhoid infection in the patient submitted to serum treatment.

We wish to acknowledge our thanks to Dr. A. Felix, of the Lister Institute, for the different typhoid strains and samples of sera put at our disposal, and for his personal interest in these investigations.

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HAY FEVER: THE SKIN-REACTIVE POTENCY OF PROTEIN AND CARBOHYDRATE FRACTIONS OF TIMOTHY POLLEN.

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IT was thought originally that the pollen idiotoxins were of protein nature, a belief based, perhaps, on insufficient experimental evidence. In 1925 Grove and Coca reported their failure to inactivate ragweed pollen extracts by digestion with proteolytic enzymes, and concluded that the idiotoxin of ragweed was probably non-protein. Similar opinions were expressed by Black (1925) and Black and Moore (1926). Black (1931, 1932) obtained from ragweed pollen a carbohydrate fraction (containing about 6 p.c. of nitrogen) which elicited specific skin reactions in sensitive individuals, and he concluded that this fraction was the active factor in ragweed. Meanwhile Loeb (1930) and Bouillene and Bouillene (1931) succeeded in inactivating ragweed and other pollen extracts by prolonged digestion with proteolytic enzymes, and they concluded that the idiotoxins were either protein or closely associated with protein. Then Stull and his co-workers (1930–33) isolated albuminous proteins from ragweed, timothy and certain other grass pollens, which were reported to elicit specific skin-reactions in dilutions of approximately 1–1,000,000. These proteins were found to completely desensitize active sera in passive transfer sites to whole pollen extract, and it was concluded that they were the only active hay-fever constituents in their respective pollens. Stull described

also carbohydrate fractions (containing, however, 5–6 p.c. of nitrogen), which gave skin reactions in a number of sensitive patients, but he concluded that their activity was due probably to the presence of small amounts of the highly active albuminous proteins. Gough (1932) prepared a highly purified carbohydrate from timothy pollen, but Freeman and the present writer (1932) failed to obtain skin reactions with this carbohydrate, and found that it had no desensitizing effect on active sera in passive transfer sites.

The present communication reports preliminary experiments on the skin-reactive potency of protein and carbohydrate fractions of timothy pollen.

CHEMICAL METHODS.

250 c.c. of stock 10 p.c. (100,000 units per c.c.) alkaline-saline extract prepared from acetone-defatted timothy pollen were employed. This slightly cloudy extract was cleared by the addition of $N/10$ NaOH sufficient to bring the reaction to approximately pH8, left in the ice-chest overnight and then passed through a Seitz filter. The clear filtrate was made slightly acid with acetic acid and poured into 5 volumes of ethyl alcohol; a heavy precipitate was thrown down and the mixture was returned to the ice-chest. After 48 hours the major portion of clear supernatant was poured off and discarded. The remainder was centrifuged and the dark brown sticky deposit washed with 90 p.c. alcohol and this removed *in vacuo*. The deposit was dissolved in 50 c.c. of water by the addition of 0·1 c.c. of $N/10$ NaOH. A slight sediment was removed by filtration, and the clear filtrate acidified with a few drops fo acetic acid and placed in a water-bath at 100° C. for 4 minutes. A heavy flocculent protein precipitate formed and was collected by centrifuge.

(1) *The protein precipitate.*—This was washed with water which had been slightly acidified with acetic acid and then dissolved in saline by the addition of about 0·2 c.c. of $N/10$ NaOH. Solution took place readily at room temperature giving a slightly cloudy fluid. This was filtered clear and poured into 5 volumes of ethyl alcohol. The resulting precipitate was collected by centrifuge and redissolved in 5 c.c. of water by the aid of a little alkali. Dilute acetic acid was then added drop by drop until a precipitate appeared. More acid was cautiously added till no further precipitate formed. The mixture was then centrifuged. (*a*) The precipitate ("G") was redissolved in 5 c.c. of water plus a little alkali. Acid precipitation and re-solution was carried out a second time. (*b*) The supernatant ("A") was filtered clear. Both G and A solutions were dialysed through cellophane 600 against distilled water for 48 hours. Solution A remained clear. G precipitated as the result of the dialysis and was cleared by the addition of a trace of alkali. Both solutions were then poured into 6 volumes of ethyl alcohol and the precipitates collected and dried *in vacuo* over calcium chloride. The yields of A and G were 16 mg. and 4 mg. respectively.

(2) *The carbohydrate-containing supernatant* from the acid-heat precipitation of the proteins was poured into 6 volumes of ethyl alcohol and a heavy flocculent precipitate formed. After standing in the ice-chest for a few hours the supernatant was decanted and discarded. The gummy precipitate was drained, and, after removal of the alcohol, dissolved in 10 c.c. of water plus

a trace of alkali. Trichloracetic acid was then added to remove any non-heat-precipitable proteins present and the mixture placed in the ice-chest. A flocculent precipitate settled out. The cloudy supernatant was filtered through paper, but as it remained cloudy it was returned to the ice-chest for a further 18 hours and was then passed through a small Seitz filter. The clear filtrate was poured into 10 volumes of dry redistilled acetone and the precipitate collected by centrifuge. This acid gummy precipitate was drained, dried and extracted with 5 c.c. of water, and sufficient alkali was added to bring the reaction to about pH5. The solution was then filtered clear and dialysed through cellophane 600 against distilled water for 48 hours. It was then poured into 10 volumes of acetone and the resulting precipitate collected by centrifuge. The precipitate (" C ") was dried *in vacuo* over calcium chloride. The yield was 26 mg.

IMMUNOLOGICAL METHODS.

Skin tests.—1–1000 solutions of A, G and C in carbol-saline and 20,000 units per c.c. timothy pollen extract were used for the comparative tests described below. For these tests the prick method (Harley, 1933) was employed, and the average diameters of the reaction wheals calculated from planimeter readings of the wheal tracings as described previously (Harley, 1937). The skin-reactive titres of the fractions were estimated by the reactions to intradermal injections of 0·025 c.c. of suitable saline dilutions of the 1–1000 solutions. Carbol-saline was used for control tests by both methods. All skin reactions were recorded 12 minutes after making the tests. The Noon pollen unit system is herein employed.

RESULTS.

Chemical Properties of the Fractions A, G and C.

Fraction A (heat-precipitable, non-acid-precipitable).—This was readily soluble in saline and in water containing a trace of alkali. In 1 p.c. solution it gave the following colour and precipitation tests characteristic of protein: biuret, ninhydrin, xanthoproteic, Millon, glyoxylic acid, lead acetate and salicylsulphonic acid. The Molisch reaction was a weak positive. Quantitative elementary micro-analysis gave the following figures: Carbon, 54·5 p.c.; hydrogen, 7·6 p.c.; nitrogen, 12·5 p.c.; and residue, 3·1 p.c. These quantitative and qualitative data, together with the method of preparation, indicate that fraction A consisted chiefly of denatured protein of albumin type.

Fraction G (heat-precipitable, acid-precipitable).—This was soluble in saline containing a little alkali. A 1 p.c. solution gave the above protein colour and precipitation tests and a slight Molisch reaction. There was insufficient material for micro-analysis, but this fraction was considered to be denatured protein of globulin type.

Fraction C.—This was readily soluble in water. The Molisch reaction was positive in high dilution. In 2 p.c. solution it gave a faint biuret reaction and a weak ninhydrin reaction. Fehling's solution was not reduced. After hydrolysis with sulphuric acid Fehling's test showed the presence of considerable quantities of reducing sugars. The nitrogen content of this fraction was

3·45 p.c. It was concluded that fraction C consisted chiefly of carbohydrate, together with a small amount of protein.

Skin Reactive Properties of Fractions A, G and C.

All three fractions in dilutions of 1–1000, when tested by the prick method, elicited immediate skin reactions in grass-pollen-sensitive patients exactly similar to those produced by whole timothy extract, and failed to do so in normal non-sensitive individuals. The relative skin-reactive potencies of the fractions were examined by two methods. The first was the comparison of the size of the prick test reaction wheals to 1–1000 solutions of the three fractions, and the second was the determination of the highest dilution of each fraction capable of producing reactions by the intradermal test.

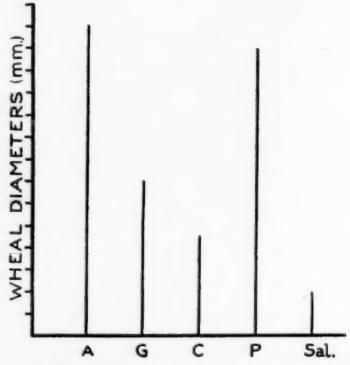

FIG. 1.—Skin-reactive potency of 1–1000 solutions of fractions A, G and C, and of whole timothy pollen extract (20,000 units per c.c.). Average wheal diameters (prick test) in a group of six hay-fever patients. A = fraction A 1–1000 solution; G = fraction G 1–1000 solution; C = fraction C 1–1000 solution; P = whole timothy pollen extract (20,000 units per c.c.); Sal. = normal carbol-saline.

Fig. 1 gives the average diameters of the prick test reaction wheals of a group of hay-fever patients to 1–1000 dilutions of A, G and C, and to whole timothy pollen extract. It was estimated from these data that the skin-reactive potency of fraction A was approximately 15 times that of fraction G and 20 times that of fraction C.

This estimate of the relative potencies of the fractions was confirmed by the results of their reaction titre determinations. Employing the intradermal technique with doses of 0·025 c.c. of test fluid, it was found that fraction A in a dilution of 1–5,000,000 regularly elicited immediate reactions in patients sensitive to whole timothy pollen extract, and occasionally did so in a dilution of 1–10,000,000. The titres of fractions G and C were about 250,000.

The desensitizing effect of fraction A on pollen-sensitive sera was examined by the passive transfer method. Unit volumes of potent serum were mixed with unit volumes of (i) saline, (ii) whole timothy pollen extract (250 units per c.c.), and (iii) fraction A in 1–50,000 solution. The mixtures (0·15 c.c. quantities) were injected intradermally in normal non-sensitive individuals

susceptible to passive transfer. After 48 hours 0·025 c.c. of timothy pollen extract (2000 units per c.c.) was injected into each mixture site through the original needle track and the ensuing reactions recorded 12 minutes later. It was found that the sites of the whole extract + serum and fraction A + serum mixtures did not react to the test injection of whole extract, while the control saline + serum sites reacted in every case. It was concluded that fraction A was capable of desensitizing active serum to whole timothy extract, and that the skin-reactive property of this fraction was immunologically the same as that of the whole extract.

DISCUSSION.

The above results indicate that fraction A contained the timothy pollen idiotoxin in high concentration, the specific skin-reactive titre being as great as 1–5,000,000, which compares favourably with the potency of histamine as tested by the same technique. While it is not claimed that fraction A is necessarily a single chemical entity, the chemical data are strongly suggestive that it consists practically entirely of protein of albumin type. From the immunological standpoint, therefore, it would seem reasonable to assume that this protein complex represents the idiotoxin of timothy pollen. The lower grade activity of fraction C (carbohydrate), together with the chemical evidence of the presence of small amounts of protein, and the previous failure to demonstrate skin activity with the purified carbohydrate prepared by Gough in 1932, suggest that this activity is due to the protein content of this fraction. Similarly, with fraction G (globulin) it is considered likely that its activity is due to small amounts of the highly active albumin. In these preliminary experiments there was insufficient yields of C and G to permit of further attempts at purification in order to settle this question concerning their skin activity.

These conclusions are in general agreement with those of Stull and his collaborators, who worked with non-denatured albumin and globulin fractions prepared by salting-out methods.

However, it is evident from the method of preparation that fraction A represents the idiotoxin protein complex in a state of considerable denaturation, and it is well known that the immunological properties of native proteins are usually considerably affected by even slight denaturation. Here we have this denatured albumin with a skin-reactive potency of 1–5,000,000, which is at least as great as that of the non-denatured albumin isolated by Stull, even when allowance is made for possible differences in skin test technique. The hypothesis which immediately presents itself is that the specificity of the idiotoxin is due to a comparatively stable group attached to or forming part of the protein complex, and that this group is activated by the remaining protein irrespective of whether the latter is native or denatured. The study of synthetic antigens, prepared by linking simple chemical groups to proteins, has shown that the immunological specificity of the complex antigen is frequently determined more by the nature of the attached groups than by the proteins to which they are coupled, even though the action *in vitro* of the simple substance on the antibody to the complex antigen may be no more than an

inhibition effect on the precipitation between complex antigen and antibody. The work of Benjamins, van Dishoeck and German (1935) on the pressure filtration of pollen extracts through protein-tight celloidin membranes supports this hypothesis. These investigators reported that protein-free filtrates were capable of producing reactions in hay-fever subjects, and further, that when such filtrates were diluted so that they failed to react in the hay-fever skin, they could be rendered active by admixture with certain protein and other colloids such as serum or egg albumin. These results suggest that the specific activity of the idiotoxin is due to a relatively small molecule, which needs a larger complex for its full activation.

Though it remains for future research to attempt the isolation and examination of this hypothetical haptene, one is almost tempted to speculate on its possible relationship to the R.I.S. (reaction-inhibiting substance) found in the serum of hay-fever patients after pollen extract therapy (Harley, 1937).

SUMMARY.

1. Protein and carbohydrate fractions of timothy pollen were prepared.

2. Fraction A (denatured protein of albumin type) produced specific skin reactions to a dilution of 1–5,000,000.

3. Fraction A desensitized hay-fever serum in passive transfer sites to whole timothy extract.

4. It was concluded that fraction A represents the idiotoxin of timothy pollen in a denatured but highly active state.

5. The skin-reactive potencies of fractions G (denatured protein of globulin type) and C (carbohydrate plus a small amount of protein) were considerably less than that of fraction A, and it was considered that their activity was due probably to the presence of small amounts of the active albumin.

6. The question of the specificity of the timothy idiotoxin being due to a comparatively small and stable grouping attached to and activated by a protein complex is discussed.

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OBSERVATIONS ON *B. WELCHII* TYPE D: ITS OCCURRENCE IN NORMAL ANIMALS AND THE VARIATION IN ANTIGENIC CHARACTER OF ITS TOXIN.

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In recent years various types of *B. welchii* differing in their toxic components have been described in connection with diseases among animals, *e. g.* "lamb dysentery" (Dalling, 1926), "struck" (McEwen, 1930), "infectious entero-toxæmia" (Bennetts, 1932), "pulp kidney" disease (Gill, 1933). The presence of three antigenic factors, W, X and Z, in the toxins of the *B. welchii* and the division of this species into four types, A, B, C and D, according to the distribution of these components was demonstrated by Wilsdon (1932 and 1933). He found that factor W was produced to a certain extent by types B, C and D, and was the only factor contained in type A toxins. Factor X was found in types B and D, and factor Z was present and predominant in types B and C. Each type embraced organisms isolated from a particular source.

With the exception of a number of strains of human origin (Borthwick and Gray, 1937) the type or types normally present in the alimentary tract of man and animals have received little attention. In this paper the types of *B. welchii* isolated from the intestine of guinea-pigs, rabbits, dogs and human subjects are recorded. Difficulty was experienced with many of these strains in obtaining toxin of sufficient strength for the determination of the type, and a preliminary study was made of the optimal conditions for toxin production.

Certain observations suggest that the production of particular toxic constituents may vary. The author (Borthwick, 1935) found that type A antisera protected against infection in guinea-pigs by both types A and D, and postulated that in these animals the X factor of type D toxin might not be produced. In addition, changes in the toxic components of types kept in culture have been noted. Thus, a lamb dysentery culture (type B) lost the power to produce the X factor (Dalling, 1934); a type D culture produced the Z factor (Dalling, 1932; Mason, 1935); also a highly toxic strain became completely atoxic so far as could be ascertained (Walbum and Reymann, 1933). In view of the foregoing observations particular attention has been paid in the present investigation to the stability of strains as regards their production of these antigenic factors.

Source and isolation of strains.

The standard *B. welchii* types A, B, C and D had been kept as stock cultures for some considerable period. They were originally received from Dr. P. Bruce White (type A) and Dr. R. A. O'Brien (types B, C and D). Other strains were isolated from the intestines of guinea-pigs, rabbits, dogs and human subjects. The material from the human subject was received from Dr. W. G. Millar, pathologist to the Royal Infirmary of Edinburgh. A specimen of the contents of the large intestine was inoculated into 5 p.c. serum peptone water and incubated anaërobically

at 37° C. for 24 hours. In this medium *B. welchii* produced spores which survived subsequent exposure to a temperature of 65° C. for half an hour. Glucose-blood-agar plates (Zeissler, 1930) were then inoculated, and after incubation for 24 hours under anaerobic conditions single colonies of typical appearance (Zeissler, 1930) were picked off into Robertson's meat medium. Further identification of *B. welchii* was made by the production of acid and gas in glucose, lactose, saccharose and maltose, and the absence of fermentation of mannitol and salicin, also by the production of a stormy-clot reaction in milk. Cultures were maintained in meat medium.

Production and preparation of toxin.

Dried toxin was used for certain experiments. The preparation of this has been described in previous papers (Borthwick, 1935; Borthwick and Gray, 1937). The procedure involved in preparation was, however, too elaborate for preliminary use with the large number of strains in the present work and unfiltered fluid cultures were utilized. The presence of toxin was determined by the death of mice within 24 hours of intravenous injection of 0·5 c.c. of culture. 20 to 22 hours' incubation at 30° C. was found to be favourable for toxin production (Walbum and Reymann, 1933). Many of the strains isolated produced little or no detectable toxin, and three different media were tested before one was discovered which was favourable for the formation of toxin. The media tested were: (1) Cooked meat with a supernatant fluid of 1 p.c. lab. lemco, 1 p.c. Hopkins and Williams peptone and 0·5 p.c. sodium chloride. In this medium the standard type strains produced active toxins, but the majority of the newly isolated strains either produced no toxin, or amounts which were not detectable. (2) Meat medium with a supernatant fluid of ox heart extract; and (3) as (2), but with the addition of 1 p.c. Witte peptone. Active toxins were obtained from many of the strains with media (2) and (3); the presence of Witte peptone gave considerably increased yields. There were, however, still many strains from which no detectable toxin could be obtained, and others showed considerable fluctuation in toxin-producing power in different batches of similar media. It was thought that the formation of acid might have destroyed the toxin or inhibited its production, and the H-ion concentration of cultures after incubation was, therefore, examined. It varied little, however, from pH 6, *i. e.* the reaction at which *B. welchii* toxins are most stable (Walbum and Reymann, 1933). As a further precaution against the possible destruction of toxin by acid a 1 p.c. buffer solution of sodium dihydrogen phosphate was included in the media. The addition of the buffer in these media neither increased nor stabilized the yield from strains which already produced toxin, nor assisted toxin-production by strains which yielded no detectable toxin. (4) Veal phosphate broth containing 1 p.c. de Fresne's peptone, 0·1 p.c. glucose, 0·2 p.c. potassium dihydrogen phosphate and cooked meat. Toxins of greatly increased activity were obtained in this medium, and many strains which had not done so before now yielded active toxin. During the foregoing tests it was observed that the use of absolutely fresh meat and meat extract in the preparation of the medium almost eliminated fluctuation in toxin production and, in addition, the optimal yield of toxin was obtained.

Antitoxin.

B. welchii standard type antitoxins A, C and D were obtained from Dr. O'Brien of the Wellcome Laboratories.

Identification of types.

A method recommended by Dr. W. S. Gordon, of the Moredun Research Institute (private communication), was adopted for typing the majority of strains. The average lethal dose for mice of toxic culture was determined. Four to five average lethal doses were then allowed to stand at room temperature for one hour in the presence of 0·1 c.c. standard *B. welchii* type antisera, A, C, D, a mixture of C and D (representing B) and normal serum as a control. 0·5 c.c. of the mixtures were then injected intravenously into each of two to four mice. Survival of the mice for 24 hours was accepted as proof of the neutralization of the toxin. The identification of the toxins is illustrated as follows:

Determination of Type of B. welchii.

Mice Injected with Toxin-antitoxin Mixtures.

Antitoxin. (0·1 c.c.).	Toxin (0·5 c.c.). Type A.	Type B.	Type C.	Type D.
A	S	+	+	+
C	S	+	S	+
D	S	+	+	S
C and D	S	S	S	S
Normal serum	+	+	+	+

+ = Death within 24 hours. S = Survival after 24 hours.

Dried toxin was prepared from a number of the more active toxin-producing strains and used to determine the type. Four A.L.D. for mice in 0·25 c.c. saline was kept for 1 hour at room temperature with 0·25 c.c. type antisera A, C, D and C plus D (representing B) at a dilution of 1 in 5, also normal serum as a control. As in the previous tests, 0·5 c.c. doses of the mixtures were then injected intravenously into mice and neutralization determined by the survival or death of the mice.

TABLE I.—*Types of* B. welchii *Isolated from the Intestine of Normal Animals.*

Species.	Animals from which strains were isolated. Identification number.	Number of strains.	Type of *B. welchii*.
Guinea-pigs	1	2	Type D.
	2	2	
	3	2	
Rabbits	1	4	Type A.
	2	5	
Dogs	1	4	Type A.
	2	2	
	3	3	
	4	4	Type D
	5	4	3 Type A, 1 Type D
	6	3	Type A.
Human	294	3	Type A.
	297	2	
	298	3	
	317	4	
	320	2	
	18751	2	

A list of the types determined by these methods is given in Table I. All strains isolated from guinea-pigs were type D, and those from rabbits and from the human subject type A. Both types A and D were found in dogs. On no occasion was *B. welchii* other than types A or D isolated. This does not, however, exclude the possibility of the presence of other types in these animals. It will be observed that more than one strain from a single animal or person

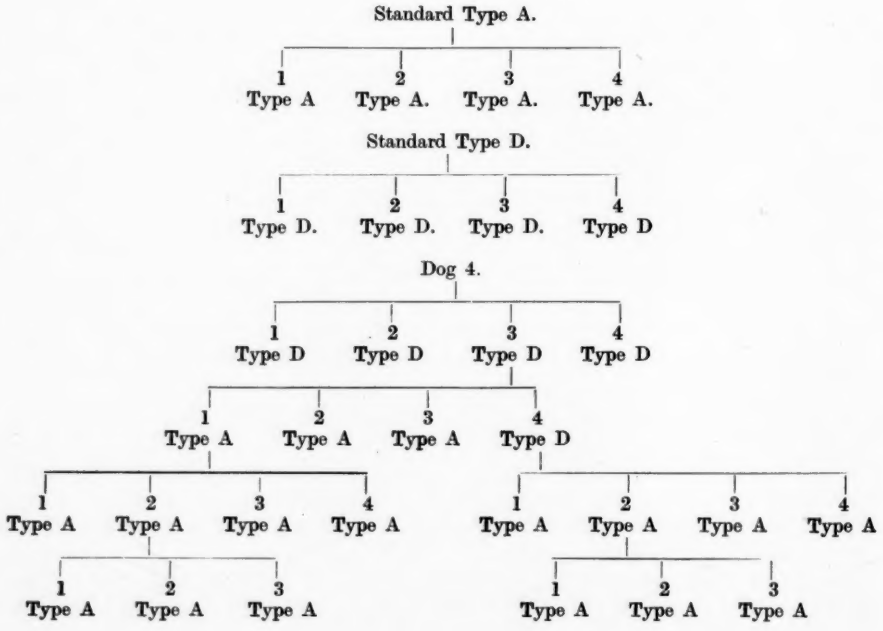
was usually tested, and, in general, these were of the same type. The finding, however, of three type A strains and one type D strain from dog 5 shows that this is not invariable.

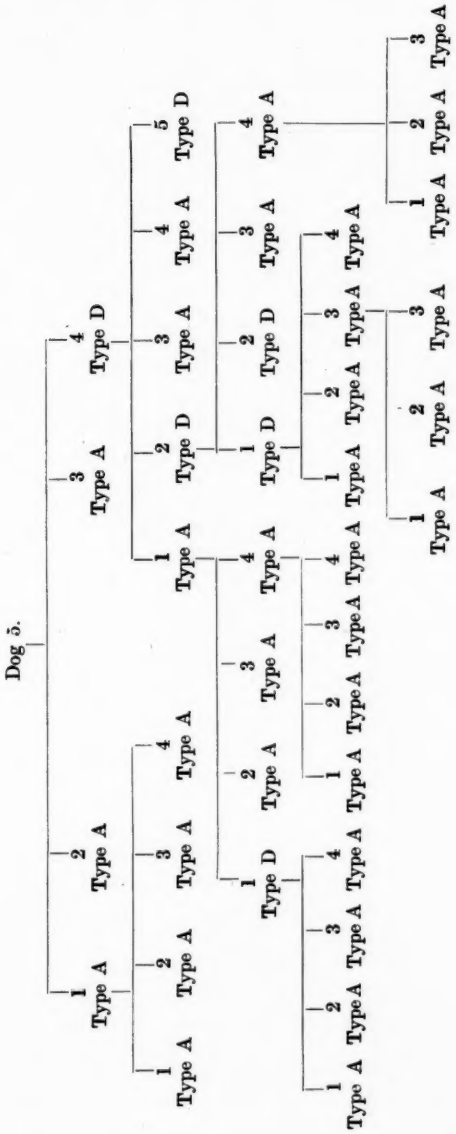
Stability of types.

On account of the diversity in the types of *B. welchii* isolated from dogs, it was decided to investigate the antigenic stability of strains from these animals. For this purpose those strains isolated from dogs 4 and 5 were selected. In addition, standard types A and D were studied as controls.

The strains were plated on glucose blood agar, and after incubation for 24 hours several single colonies were subcultured and toxin prepared from each. The type of toxin was then determined by the first method described (see identification of types). When any variation in type had occurred, this procedure was repeated. The results are illustrated in the "pedigrees" below.

It will be seen that standard types A and D, which had been kept as stock cultures for some years, were antigenically stable. On the other hand, single-colony cultures from strains freshly isolated from dogs varied and showed a tendency towards simplification. Thus type A strains were not infrequently derived from type D colonies, and there was only one example of a type D strain derived from a type A colony (dog 5, 4), and in this instance the type A colony had originated from a type D strain.




Dog δ.
1 Type A
2 Type A
3 Type A
4 Type D
1 Type A
2 Type A
3 Type A
4 Type A
1 Type A
2 Type D
3 Type A
4 Type A
5 Type D
1 Type A
2 Type A
3 Type A
4 Type A
1 Type D
2 Type A
3 Type A
4 Type A
1 Type A
2 Type D
3 Type A
4 Type A
1 Type A
2 Type A
3 Type A
4 Type A
1 Type A
2 Type A
3 Type A
4 Type A
1 Type A
2 Type A
3 Type A

An attempt was made to alter the antigenic characters of *B. welchii* standard type D by incubating equal amounts of culture in cooked meat and standard type D antisera for 24 hours at 37° C. As in the tests for the stability of types, the culture was then plated on glucose-blood-agar and the type of toxin prepared from single colonies investigated. Type D only was found.

DISCUSSION.

The preceding experimental findings suggest that antigenic variation may occur among the members of the *B. welchii* group. Factors influencing this variation have not yet been ascertained. It seems possible, however, that environmental conditions in the alimentary tract are responsible for the finding of only one type of *B. welchii* in many animals. Thus numerous strains of human origin tested were all type A (Borthwick and Gray, 1937 ; and this paper). *B. welchii* type A and occasionally type D were isolated from dogs. All these type D strains lost the antigenic factor " X " in culture and retained only the type A factor " W ", thus becoming typical A strains. The development of the " X " factor in the alimentary tract may depend on environmental conditions. In this connection, however, it may be noted that the medium used was not inhibitory to the production of the " X " component, as on primary isolation many strains actively produced the " X " factor. This suggests that an actual alteration in the antigenic character of the strains occurred.

The failure to produce any antigenic change in the standard type D strain suggests that cultures which have been kept for some considerable time as stock laboratory cultures become stabilized.

In considering the findings reported in this paper, the question would arise whether the single colonies investigated constituted only one type. It might be suggested that the cultures which showed antigenic changes contained a mixture of types. Other than by single-cell culture there is no means of excluding this possibility. It seems probable, however, that the cultures were pure, as they were originally isolated from single colonies, and on all subsequent occasions the identification tests were made with cultures which had been grown from single colonies. Moreover, on account of its simpler antigenic character, type A would tend to be obscured in a mixed culture of types A and D. The reverse, however, was observed.

SUMMARY.

1. Veal phosphate broth with de Fresne's peptone and meat favours the production of *B. welchii* toxin. The use of fresh meat and meat extract increases the yield of toxin and decreases fluctuation in toxin production.

2. Strains of *B. welchii* type D were isolated from guinea-pigs, type A from rabbits and the human subject, and types A and D from dogs.

3. Standard strains of *B. welchii* types A and D have been found to be antigenically stable. *B. welchii* strains isolated from dogs have proved variable in the antigenic characters of their toxins.

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THE CULTIVATION OF RABIES VIRUS IN TISSUE CULTURES.

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The medium introduced by Maitland has, with various modifications, proved useful for the cultivation of certain viruses and *Rickettsia*. This success has, however, been limited to dermotropic viruses. Efforts to cultivate neuro-viruses have until recently yielded doubtful or negative results. In the present paper we report on attempts to cultivate a strain of fixed rabies virus *in vitro*. Noguchi (1913) reported that he cultivated this virus in his medium for growing spirochætes. These results could not, however, be confirmed. Levaditi (1914) found that infected spinal ganglia, kept in plasma at 37° C., remained infective for 53 days. Stoel (1930) reported the successful cultivation of a street virus in Borel cultures ; he used the whole head or brains of chicken embryos and carried the virus through five subcultures during a period of 25 days. Waldhecker (1935), on the other hand, obtained wholly negative results with two different strains of fixed virus and two strains of street virus. He employed different media, among them also the Maitland medium. While our work was in progress, Webster and Clow published a preliminary report (1936) on the successful cultivation of two strains of rabies virus which had been maintained in mice. These authors used a modified Maitland medium with monkey serum and mice embryo brain tissue and passed the virus through 16 subcultures. A more extended report has appeared recently (1937).

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In our experiments, carried out before the appearance of the preliminary report by Webster and Clow, we tested a variety of media and finally obtained definite evidence of multiplication in a plasma medium to be described below. Subsequently we also used the method described by these authors. The object of this paper is to report on the successful cultivation of our strain of rabies virus under various conditions.

The strain of virus used throughout these experiments was kindly furnished by Dr. Krikorian, of the Palestine Department of Health. It is supposed to be derived from the original Pasteur virus, and has been maintained in the Health laboratories for many years by rabbit passage. An intracerebral inoculation produces in rabbits a typical infection beginning with fever on the 4th or 5th day; paralysis sets in on the 6th or 7th day, and death occurs on about the 10th day. The brain, removed on the 7th or 8th day, is, as a rule, infective up to a dilution of 10^3–10^4.

CULTIVATION IN PLASMA MEDIUM.

Methods.

Medium.—One c.c. of chicken plasma, diluted 1 : 3 with Tyrode solution, was placed in a 25 c.c. Erlenmeyer flask. Small bits of rat or mouse embryo brain (which had been treated with virus) were placed in the plasma and a drop of embryo extract added to cause coagulation of the plasma. The infected bits of tissue were thus embedded in a thin layer of plasma. The plasma was then covered with 1·5 c.c. Tyrode-serum, consisting of 9 parts Tyrode solution and 1 part rabbit serum. The flasks were then stoppered with rubber stoppers and incubated at 37° C.

Virus.—The virus was obtained from rabbits. An infected rabbit was killed on the 7th or 8th day after infection, or a day or two after paralysis had set in. The brain was removed, placed in a sterile mortar, and ground with sand, saline being added slowly so as to give a 10 p.c. suspension.

Embryo tissue.—The infected bits of embryo brain were prepared as follows: Mouse embryos were mostly used, two or three days before birth. The pregnant animal was etherized and the embryos removed aseptically. The embryo brain was removed into a sterile Petri dish and macerated with a fine sterile scissors. About three drops of the virus suspension were added to a number of fragments of the macerated embryo brain in the Petri dish, and allowed to stand for 10 to 15 minutes. The fragments were then taken up on a platinum spatula and transferred to the plasma in the Erlenmeyer flasks. Five to six fragments, weighing about 5·0 mg., were placed in each flask.

Serial transfer.—Subcultures were made after incubation for 4–5 days at 37° C. The fragments of tissue from two flasks were removed from the plasma into a sterile glass mortar and ground into a fine pulp. A few drops of this emulsion were then added to fresh mouse embryo brain fragments and inoculated, as above, into fresh culture flasks.

The initial amount of virus inoculum in the culture was very small. Control inoculations into rabbits of 0·2 c.c. of the undiluted ground-up culture before incubation as a rule failed to infect. Each culture was tested by intracerebral

inoculation into rabbits. The cultures, as well as the brains of the infected rabbits, were always tested for sterility in broth and on agar. The small amount of virus in the culture and the subsequent positive infections furnish, therefore, clear evidence of multiplication.

Results.

The results showed that the virus multiplied in this medium. In one experiment seven serial passages were made. The first culture of this series, after an incubation of four days, when inoculated into rabbits, produced paralysis only after twelve days, whereas subsequent passages produced typical paralysis after six days, with constant results. Considering the very minute quantity of virus added to the culture medium, it is apparent that in the 7th subculture, the initial amount of virus had been diluted at least a billionfold.

That actual multiplication occurred in this medium is also evident from the following experiments: In three separate experiments the initial culture, before incubation, failed to infect rabbits inoculated intracerebrally with 0·2 c.c. of the emulsified culture. After incubation for 4 days at 37° C. the emulsified cultures of the same series produced a typical infection in rabbits, with paralysis in 6 days. In the second subculture, dilutions of the culture suspension of 1 : 100 also produced a typical infection with paralysis in 6 days. The same results were obtained with the third subculture.

Since the cultures were not always uniformly positive, we subsequently modified the medium somewhat. Instead of 1·0 c.c., 1·2 c.c. of plasma-Tyrode were added to the flask, and instead of rabbit serum, human serum was used. The rest of the procedure was the same. In this medium we carried the culture through seven transfers, when it was discontinued. The initial culture infected rabbits when undiluted. The 5th to 7th transfers were infective in dilutions of 1 : 100, but not in dilutions of 1 : 1000.

The results prove that it is possible to propagate fixed rabies virus *in vitro*. The procedure, however, was not entirely satisfactory from a practical point of view. At times we experienced difficulty in carrying on the cultures through successive subcultures. The reasons for the loss of cultures appear to be twofold: In the first place, so little virus is present at the outset that unless conditions for development are ideal, it is apparently lost before multiplication can begin. In the second place we found that the quality of the chicken plasma is very important—unless the plasma is satisfactory, the embryo tissue fragments disintegrate and no growth occurs. Consequently, although the method is suitable for demonstrating multiplication of virus, and has the advantage that only small amounts of tissue are used, it is not satisfactory for continued mass propagation.

CULTIVATION BY THE METHOD OF WEBSTER AND CLOW.

On the appearance of the preliminary report by Webster and Clow, we attempted to apply their technique to the cultivation of our strain of fixed virus. Their technique differed from ours in three essential points: they did

not use plasma ; they used very large inocula ; and monkey serum was used in place of rabbit serum. We have succeeded in our attempt, and in the process of adapting their technique we have made some observations on the influence of different sera which are worth recording.

In all respects, except the variation of the sera, we followed closely the procedure outlined by Webster and Clow. In 50-c.c. Erlenmeyer flasks were placed 3·6 c.c. Tyrode solution, 0·4 c.c. serum and one or two drops of a thick suspension of mouse-brain embryo. This medium was then inoculated with 1 c.c. of a 1·0 p.c. virus suspension prepared by grinding up the brain of an infected rabbit in saline. The flasks were closed with rubber stoppers and incubated, some at 30° C. and some at 37° C. Subcultures were made after 3 or 4 days by emulsifying the contents of two flasks and inoculating 1 c.c. into fresh media. At the same time rabbits were inoculated to ascertain the virulence of the cultures.

Rabbit serum.—The first series of cultures were made with rabbit serum, since it had proved satisfactory for the growth of the virus in the plasma medium. In no case, however, did we succeed in carrying the cultures beyond the third passage with a total incubation time of 9 to 12 days. As so large an inoculum was used, the virus in the third subculture was in all probability carried over from the original inoculum.

Monkey serum.—We then proceeded to use monkey in place of rabbit serum. No difficulty was experienced in carrying the virus through 10 subcultures over a period of 45 days. The first two cultures were infective only when undiluted. From the third passage onward a 1 : 100 dilution produced typical infections with paralysis on the 6th to 7th day after the inoculation. The titre of the cultures incubated at 37° C. remained stable between the 4th and 7th day of incubation. Thereafter their virulence decreased so that on the 15th day only the undiluted culture was still infective, and on the 20th day the cultures were no longer infective. Multiplication and survival at 30° C. were essentially the same as at 37° C.

Human serum.—Since fresh monkey serum is not always readily available, we repeated these experiments with human serum. Normal serum as well as sera sent to the laboratory for diagnostic purposes were used. The results were entirely satisfactory. Often the human serum caused a clumping of the fragments of embryo tissue, but this did not adversely affect the multiplication of the virus. We have now carried the strain through 15 subcultures during a period of 10 weeks without diminution in virulence. Rabbits inoculated with cultures developed a typical infection, paralysis setting in on the 6th or 7th day. These cultures were also infective in a dilution of 1 : 100. Broth and agar cultures of the brain of the infected rabbits were sterile. Serial passages, with typical effects, were also made from rabbits infected with the tissue culture virus. The survival of virus in these cultures kept at 37° C. was the same as in those made with monkey serum.

Effect of formalin.—Preliminary experiments have been made on the inactivation of the virus by formalin. It was found that in a final concentration of 1 : 1000, formalin inactivated the cultures completely after 1 hour at 37° C. followed by 48 hours in the icebox. Immunization experiments with inactivated culture virus are now in progress.

SUMMARY.

Experiments are reported indicating that it is possible to cultivate fixed rabies virus in media containing rat or mouse brain embryo.

Serial subcultures were obtained in a medium consisting of chicken plasma covered with Tyrode-serum. In this case rabbit serum may be used, although human serum is better.

The procedure reported by Webster and Clow is more satisfactory for mass cultures. In this medium no growth was obtained with rabbit serum, but successful serial passages were made in media containing either monkey or human serum.

The virus cultures incubated at 30° C. or 37° C. increase in virulence up to about the 6th or 7th day. Thereafter the virulence declines and disappears by the 20th day.

Formalin (1 : 1000) inactivates the cultures if kept one hour at 37° C. and then for 48 hours at icebox temperature ; the rate of inactivation appears to depend on the concentration of the formalin.

We want to take this opportunity to express our appreciation to Dr. Doljansky, of the Cancer Laboratories, for his kind co-operation, particularly in supplying us with chicken plasma and embryo extract.

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INFLUENZA VIRUS INFECTION OF RATS AND GUINEA-PIGS.

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IN a previous communication (Stuart-Harris, 1936), the transmission to hedgehogs of influenza virus previously passed through ferrets and mice was described. The disease in the hedgehog resembled that of influenza in the ferret, being a contagious infection of the respiratory tract, but was much milder and relatively symptomless. The present paper describes the results of similar experiments with influenza virus in rats and guinea-pigs. These were carried out in a search for a small laboratory animal in which influenza virus would produce a contagious disease—a search which has so far proved fruitless.

THE RAT.

Five attempts were made to establish influenza virus in the rat by the method of serial passage. The fourth of these will be described first because it was continued for the longest time and was only abandoned after 18 passages. The virus was a strain of human influenza virus (W.S.) passed through 207 " generations " of ferrets, and it was highly virulent for these animals, a high proportion of which died with consolidation of the lungs, after infection. Mouse passage had not been carried out before the inoculation of rats and, although the virus produced lung lesions in mice, it was not highly virulent for this species. The serial passage in rats was initiated by inoculating intranasally three young rats under ether anæsthesia with 0·1 c.c. each of a 5 p.c. emulsion of mixed turbinates, and portions of lung from a ferret killed on the 4th day after inoculation with the virus. The rats were killed on the 4th day after inoculation, the entire contents of the nasal fossæ were scraped out, pooled, weighed, ground with powdered pyrex glass and emulsified in equal parts of broth and Ringer's solution ; the emulsion was made up to a strength of 5 p.c. and lightly centrifuged. The supernatant fluid was cultivated on blood agar, and 0·1 c.c. was inoculated into each of three rats anæsthetized with ether. Subsequent passages were carried out on the 3rd or 4th day after inoculation in an identical manner. At times rats were inoculated with a 10 p.c. lung emulsion prepared from the lungs in a manner similar to that used for the turbinate emulsion, but the serial passage was in each case carried out with turbinate material. The turbinate and lung emulsions from each " generation " of rats were each inoculated into groups of six mice under ether anæsthesia ; the mice were killed on the 5th day and their lungs were examined for lesions. The rats were all young and averaged 40 g. in weight.

The first three " generations " of rats showed no symptoms, but the rats of the sixth generation (5th pass) had rough coats and developed a slight watery nasal discharge with crusting around the nares. Such symptoms were, however, inconstant in subsequent generations. In addition, although very rarely, a laboured type of respiration was seen. At post-mortem the nasal fossæ usually appeared normal, although slight congestion was sometimes visible, but sections of the snouts showed occasionally exudation of polymorphonuclear leucocytes into the nasal sinuses without loss of the ciliated epithelium. Such changes were inconstant and unrelated to nasal symptoms. The lungs of the rats from the fourth generation onwards showed lesions varying from purplish-brown areas of apparently solid lung near the hilum to complete brownish consolidation of entire lobes. Not all rats showed lung lesions, but these were usually present in animals which had exhibited symptoms during life. One rat only died—on the 4th day after inoculation—and the lungs of this animal showed extensive consolidation. Histologically, the affected parts of the lungs showed collapse of the alveoli, infiltration with polymorphonuclear leucocytes and monocytes, and sometimes exudation of leucocytes into the lumina of the bronchi.

The fact that influenza virus was surviving passage in the rats was established by the development of lung lesions, typical of influenza, in mice inoculated with both turbinate and lung material (Table I). However, material from the fourth generation of rats and onwards gave mouse lesions different in

TABLE I.—*Part of the Serial Passage of Influenza Virus in Rats.*

Rat Nos.	Generation.	Inoculum.	Day of killing.	Rat lung lesions.	Mouse lung lesions from rat.	
					Turbinates.	Lungs.
31–33	1	Ferret virus	4	000	002221	000000
49–51	3	Rat turbinate emulsion	4	000	212121	111100
52–54	3	Do.	12	000	0000**	000000
83–88	6	,,	3	000022	222A22	00112A
112–114	8	,,	4	A00	022222 (filtrate)	0211*0 (filtrate)
115–117	9	Rat turbinate filtrate	4	022	444***	02**01
156–161	17	Rat turbinate emulsion	3	002AA	000002 (filtrate)	02**01

Figures in the last three columns indicate the relative size of the lung lesion : 1 = small root lesion ; 2 = medium-sized lesion ; 3 = extensive lesions ; 4 = almost complete hepatization, usually a dead mouse ; * = lesion not typical of influenza ; A = abscess.

some respects from typical influenzal lesions. The lungs were greyish-red instead of purple and abscesses were occasionally present. On two occasions filtrates through gradocol membranes of A.P.D. 0·63μ and 0·64μ (Elford, 1931) were prepared after preliminary clarification through asbestos pulp, and these gave lung lesions typical of influenza in mice. But the filtrate from turbinates of the eighth generation of rats gave lesions in 5 out of 6 of the inoculated mice, while that from the seventeenth gave lesions in only 1 out of 6 mice. Thus, although the virus survived the serial passages with their accompanying dilutions, its amount, as judged by the mouse test, appeared to have decreased after many passages.

Further proof that virus was present in the rats was obtained by inoculating two ferrets with turbinate and lung emulsion respectively from the eleventh generation of rats. Both ferrets showed typical " takes ", and were subsequently immune to inoculation with W.S. virus after convalescence from the rat infection. Virus was, however, recovered only from rats during the first 4 days after inoculation, and turbinate and lung material collected 7 or more days after inoculation was invariably non-infective for mice. It was thought at first that the turbinate and lung lesions in the rat were due to the virus, but the turbinate lesions were inconstant, and cultures of the lungs with lesions always yielded a rich growth of organisms such as Gram-negative cocco-bacilli of the Pasteurella group, staphylococci and diphtheroid bacilli. Also the histology of the lung lesions in the rat was different from that of influenza in the ferret and mouse. The infiltration of the collapsed areas was largely polymorphonuclear and not mononuclear. The development of symptoms and of lung lesions in rats after several serial passages was, therefore, thought to be due probably to the action of micro-organisms.

Influenza virus was, however, definitely producing an infection in the rat, for after infection, antibodies were demonstrated in the serum by the mouse

neutralization test. Thus the pooled serum of three rats of the third generation collected 12 days after inoculation neutralized the virus up to a dilution of 1 in 10, whereas normal rat serum had no neutralizing effect on the virus. The technique used for the examination of the sera was that of Andrewes, Laidlaw and Smith (1935). Two experiments were carried out to test the possibility of transferring the virus by contact. Three normal rats were placed in the cage containing three rats inoculated with material of the ninth generation on the 2nd day after inoculation, and were allowed to mix with the infected rats for 2 days. The contact animals were killed after 2 more days, and their turbinates were emulsified and inoculated into six mice. None of the mice developed influenzal lesions. A similar result was obtained in a second experiment when rats were placed in contact with animals of the sixteenth generation.

The four other attempts to establish influenza virus in rats were abandoned after 2, 2, 3 and 10 passages, but two additional facts were elicited. Firstly, influenza virus which was highly virulent for the mouse showed no better power of adaptation to the rat than the ferret strain, although it persisted in both turbinates and lungs; secondly, an organism was recovered from a filtrate of turbinate emulsion through a membrane of A.P.D. 0·83μ by cultivation on blood-agar plates. This organism was morphologically like *Streptobacillus moniliformis*, and after subcultivation a saline suspension of the growth on blood-agar was inoculated intranasally into anæsthetized rats in varying dilutions. Some of the rats, even with a dilution of 1/125 of the suspension, showed lung lesions exactly like the lesions seen in the lungs of rats inoculated with virus-containing emulsions. As *Streptobacillus moniliformis* was shown by Strangeways (1933) to be a constant inhabitant of the nose in the strain of rats used in the present experiments, it seemed probable that the lung lesions observed in rats of the passage series were due to this organism, which was washed down into the lungs from the nose by the act of inoculation.

THE GUINEA-PIG.

The experiments which were carried out in the guinea-pig were modelled on lines similar to those in the rats. Serial passage was carried out, beginning with the strain of W.S. virus, which had then been passed through 243 ferrets, but not through mice. Three young guinea-pigs, which had recently been weaned, were inoculated intranasally under ether anæsthesia with 0·25 c.c. each of a mixed turbinate and lung emulsion from a ferret infected with influenza virus. The guinea-pigs were killed on the 3rd day after inoculation, and 10 p.c. turbinate and 20 p.c. lung emulsions were prepared. Serial passage was carried out with mixed turbinate and lung emulsions for fifteen generations and thereafter with turbinate emulsion only, and young guinea-pigs were used in each case. Mice were inoculated either with mixed emulsion or with the two emulsions separately. So far the series has been carried through 31 generations of guinea-pigs.

Table II shows that the lesions which were obtained in mice with unfiltered emulsions were rarely typical of influenza, and many of them were atypical in colour or included abscesses.

TABLE II.—*Part of the Serial Passage of Influenza Virus in Guinea-pigs.*

Guinea-pig Nos.	Generation.	Inoculum.	Day of killing.	Guinea-pig lung lesions.	Lung lesions in mice receiving—	
					Turbinates.	Lungs.
1–3	1	Ferret virus	3	122	011A**	20000A
4–6	3	Turbinate and lung emulsion	3	122	000002	—
16–18	6	Do.	3	122	******	—
28–30	10	,,	3	013	100000	00****
31–33	11	,,	3	012	001222 (filtrate)	—
70–71	20	Turbinate emulsion	1	22	AA222A	AAAA0A
97–99	23	Do.	3	013	021020 (filtrate)	000000 (filtrate)
115–117	27	,,	2	011	431222 (filtrate)	—

Figures in the last three columns indicate the relative size of the lung lesion. * = lesion not typical of influenza ; A = abscess.

Filtrates, however, gave typical lesions in mice, and more extensive lung lesions were obtained with the filtrate from the twenty-seventh generation of guinea-pigs than with those of the eleventh, fifteenth and twentieth generations. Filtrates from guinea-pig lungs were, however, negative on each occasion upon mouse inoculation, although lung lesions of atypical appearance were obtained with unfiltered emulsions. The presence of influenza virus in the filtrate of turbinates of the eleventh generation was established by the inoculation of six mice and passage from the lungs of three of them killed after 3 days to six more mice. All the latter, which were killed on the 4th day, showed lung lesions typical of influenza. A filtrate was prepared from these through a 0·63μ membrane, mixed with varying dilutions of hyperimmune anti-influenzal horse-serum IH_2 (Laidlaw, Smith, Andrewes and Dunkin, 1935), and inoculated into groups of mice. The mice receiving filtrate and serum in final dilutions of 1/10, 1/50 and 1/250 had normal lungs after 5 days, while the controls all showed typical influenzal lesions, thus proving that the serum in high dilution neutralized the virus. Two ferrets were inoculated with turbinate and lung emulsions respectively from the twenty-second generation of guinea-pigs killed on the 4th day. The ferret receiving lung emulsion remained normal and was subsequently susceptible to W.S. virus, while that receiving turbinate emulsion developed a typical attack of influenza.

Thus in the case of the guinea-pig, influenza virus survived repeated passage in series and, as judged by mouse inoculation, was increased in amount at the conclusion of the series. No evidence was obtained, however, of any increase in pathogenicity for the guinea-pig, as shown by the development of symptoms or of lesions. In the first place no nasal symptoms were observed throughout the series, and three out of four snouts examined histologically were normal. The remaining snout showed a slight exudation of polymorphonuclear leucocytes into the nasal sinuses, but without desquamation of the ciliated epithelium.

Certain of the guinea-pigs showed roughening of the fur with exaggerated respirations, and these and others which appeared normal exhibited lung lesions when killed. These lung lesions were at first thought to be due to influenza virus infection. They consisted of purplish-brown areas of solid lung, usually near the hila of the lower lobes and azygos lobes, but they varied in size and were occasionally extensive in some of the lobes. Macroscopically they resembled exactly the type of lesion found in mice infected with influenza virus, but microscopically the lesions showed infiltration with polymorphonuclear leucocytes and collapse of the alveoli. The mononuclear infiltration seen characteristically in the lung lesions of influenza in the ferret and mouse was not present. Again, no evidence was obtained that influenza virus was present in the lungs of the guinea-pigs even 24 hours after inoculation under an anæsthetic (Table III).

TABLE III.—*Survival of Virus after Inoculation of Guinea-pigs.*

Guinea-pig Nos.	Inoculum.	Time of killing.	Lung lesions in mice receiving— Turbinates. Undiluted 10 p.c.	Turbinates. 1/10.	Lungs. Undiluted 20 p.c.	Lungs. 1/10.
82–83	Turbinate emulsion 79–81	24 hours	222222	130222	000000	000000
84–85	Do.	2 days	A22224	222322	00***0	000000
86–88	,,	3 ,,	221122	002212	000000	000000
89–90	,,	4 ,,	012000	—	000000	—
137–139	Turbinate emulsion 133–135	2 ,,	2222A*	—	—	—
140–142	Do.	7 ,,	0000A0	—	—	—

Figures in the last four columns indicate the relative size of the lung lesion. * = lesion not typical of influenza ; A = abscess.

Finally the lungs with extensive lesions yielded upon cultivation on blood agar a Gram-negative cocco-bacillus, probably of the Pasteurella group, which was found to be pathogenic for the guinea-pig. Inoculation of lung emulsions containing this organism into guinea-pigs and mice produced extensive lesions, often with exudate into the pleuræ of a peculiar viscid type. A broth subculture was inoculated into three guinea-pigs under an anæsthetic. One animal died with hepatization of all lobes, and the remaining two when killed after 3 days showed lesions at the roots of the lungs exactly similar to those obtained with the virus-containing emulsions.

Influenza virus therefore produced an inapparent infection in the guinea-pig. Table III shows that after intranasal inoculation the amount of virus in the nose, as judged by mouse inoculation, increased up to 48 hours, then diminished, and was absent by the 7th day.

The infection could not be transferred by contact. Three experiments were carried out in which normal guinea-pigs were added to the cages of infected animals 24 hours after inoculation of the latter (Table IV).

TABLE IV.—*Contact Experiments in Guinea-pigs.*

Contact guinea-pig Nos.	Infected guinea-pig Nos. and generation.	Duration of contact.	Mice inoculated with turbinates from— Infected guinea-pigs.	Contact guinea-pigs.
43	40–42 (13th)	2 days	202222	000000
67–69	64–66 (19th)	2 ,,	42*0**	000000
94–96	84–90 (22nd)	2 ,,	A22224 221122 012000	00000*

Figures in the last two columns indicate the relative size of the lung lesion. * = lesion atypical of influenza; A = abscess.

Both contact and infected animals were killed and the turbinates emulsified and inoculated into mice. The period of contact was 2 days, and the contact guinea-pigs were examined for virus after a further 24 hours. The groups of six mice inoculated with turbinate emulsion from each group of contact guinea-pigs did not show lung lesions, and passage from the lungs of three of the mice killed on the 3rd day after inoculation did not produce lesions in a further group of mice. A ferret was inoculated with turbinate emulsion from one group of guinea-pigs subjected to contact, and this animal remained normal and was subsequently susceptible to W.S. virus.

Finally, infected guinea-pigs developed antibodies to the virus. The pooled sera from guinea-pigs killed 12 and 13 days after infection with virus neutralize the mouse strain of W.S. virus to a dilution equivalent to 1/125th of that of the hyperimmune horse-serum IH_2 (Table V); normal guinea-pig serum did not neutralize the virus at all. Nevertheless the convalescent guinea-pig sera did not neutralize the virus in high dilution and in this respect they resembled the convalescent rat sera, but differed from sera from ferrets convalescent from influenza (Smith, Andrewes and Laidlaw, 1935), which usually contain potent neutralizing antibodies.

TABLE V.—*Antibodies in Sera from Convalescent Guinea-pigs.*

Serum dilution.	1/2.	1/10.	1/50.	1/250.	1/1250.	Controls W.S. virus.
Hyperimmune horse-serum IH_2	—	—	000	000	222	432
Normal guinea-pig serum	433	442	—	—	—	—
Convalescent guinea-pig serum (12 days)	01*	022	022	—	—	—
Convalescent guinea-pig serum (13 days)	000	212	222	—	—	—

* = Lesion atypical of influenza.

DISCUSSION.

The result of inoculating rats and guinea-pigs with human influenza virus passed through ferrets has been to induce subclinical infections unlike the typical virus infections in either ferrets or mice. These results differ from those found by other authors. Thus Agapov (1936) inoculated rats with swine and human influenza viruses, and found that symptoms and lung lesions were produced. The infection induced by swine virus in his experiments was

found to be transmissible by contact, but in no case was rat material examined for the presence of virus by inoculation of other animals. McIntosh and Selbie (1937) obtained a virus pathogenic for ferrets and mice beginning with material from human cases of influenza, and found that this virus was highly pathogenic for both guinea-pigs and rabbits. In the present work the guinea-pig has not been found to be highly susceptible to the virulent ferret strain of human influenza virus, and Wilson Smith (unpublished observations) has found no evidence of survival of the same virus after inoculation into young rabbits. On the other hand, Burnet (1936) and Burnet and Lush (1936) described infections of the rat by louping-ill and ectromelia viruses, which resembled in some way those described above for influenza. However, inapparent infections of the rat with louping-ill and ectromelia could only be maintained for two or three generations, while in the present experiments influenza virus was passed 19 times in rats and 31 times in guinea-pigs. There was some evidence that the virus was dying out in the rat, but in the guinea-pig it was, if anything, increased in amount at the conclusion of the passage. In neither species was the infection with influenza virus recognizable clinically, nor were characteristic lesions found such as are induced by the virus in the ferret and mouse. Francis and Magill (1937) have recently described the infection of mice with influenza virus direct from human material, and in their experience and that of Andrewes (1937), the virus survives in the lungs of the mice without producing lesions for some generations before lung lesions appear. The infections of the rat and guinea-pig with influenza virus, although inapparent, differ fundamentally from such subclinical infections in mice in that repeated passage fails to cause that adaptation of the virus to its new host which is associated with the evolution of a clinically recognizable infection.

SUMMARY.

1. Influenza virus which has been passed through ferrets will produce an inapparent infection in the rat and guinea-pig.

2. Virus is recoverable from both nose and lung of the rat after inoculation under an anæsthetic, but only from the nose of the guinea-pig.

3. In neither animal is the infection transmissible by contact.

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